

nature

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Too little guidance on the PhD?

TWO-AND-A-HALF years ago the Expenditure Committee of the House of Commons put out a report on postgraduate education in Britain. Many of the recommendations stirred up strong feelings in the academic community, and there was considerable interest in how the Department of Education and Science (DES) would field this full frontal assault on the nature of the PhD. The recently published White Paper 'Postgraduate Education' (HMSO; 28p) is the rather belated reply.

The Expenditure Committee's most headline-catching suggestions were in the field of postgraduate financing. It had suggested that there should be at least an element of repayable loan in student awards above some basic minimum. This idea finds little favour with the DES and there seems no reason to resurrect it in the near future. But it was not in student finances that the strongest feelings were aroused; it was in the proposals that "pre-experience" students should be discouraged from taking non-vocational postgraduate courses but encouraged into vocational courses leading to qualifications which would be a prerequisite for entry to a career. These suggestions stemmed from the committee's view that "postgraduate education should be shaped, not by student demand alone, but principally by the needs of the economy and of society as a whole".

The Government "broadly accepts" this view in its reply; resources for postgraduate education should be provided "primarily in order to meet the country's need for trained manpower". It accordingly defends itself by pointing to the many moves that are being made to shape postgraduate education towards vocational ends, and remarks that "it would be an over-simplification to regard research training leading to the PhD as non-vocational". And yet having apparently gone a long way down the road to manpower planning in the postgraduate sector, the government then declares that "the content and balance of postgraduate training, in all its complexity, are best left to the autonomous interaction of the institutions themselves, the research councils and employers". It looks suspiciously inconsistent with manpower planning to expect that universities can work out individually how to do their bit to meet the country's

and society's need for trained manpower; and it suggests that the DES, for all its waiting to hear the views of (unnamed) relevant bodies before venturing to put its own position forward, has succeeded only in compiling a hurriedly cobbled-together case for seeming to worry about the needs of the nation while actually defending the *status quo*.

In the face of the committee's assault, the DES should have come out fighting. What evidence is there that manpower planning makes sense when it comes to controlling the intake of prospective PhD students? The Expenditure Committee found a few representatives of industry prepared to say that PhDs didn't interest them; but if they had looked in other directions they would have found industrialists prepared to say just the opposite. The Civil Service, another major employer, would equally not be of one mind. With such variability in opinions there seems little sense in trying to evolve any major strategy to switch the emphasis in postgraduate work. It is difficult to believe that there is anything like enough evidence to allow the DES to start matching postgraduate education to the "needs of the economy and of society as a whole".

A somewhat worrying omission from the DES's response is any obvious rejoinder to the committee's view that the number of pre-experience students should be "considerably" reduced. It is indeed true that the number of awards being granted for postgraduate research is slightly in decline at present, and one wonders what it will lead to. Will the downward trend be allowed to continue even if and when the economy picks up? The DES paper mentions only "some areas of study such as mathematics or non-clinical medical research" as being hindered by a student having to take a break after a first degree. It then goes on to promise a higher proportion of post-experience students. The whole looks a little ominous. Maybe we have too many young PhD students; maybe manpower planning would work. One would need more than this White Paper to be convinced, however. The DES, in its stern effort to show what it is doing for the GNP, might too easily abandon education for training.



Flashes in ashes

Allan Piper considers the biggest and most promising of five new energy projects sponsored by the International Energy Agency (IEA).

THE opening earlier this week of a London headquarters for a new National Coal Board (NCB) subsidiary will probably cause no more than a passing ripple in the whirlpool of international energy affairs. Few people can have heard of NCB (IEA Services), and fewer still will know what it represents. But its potential long-term impact on the global energy scene should not be underrated.

In the rush to develop new energy technologies, coal, once in decline as a major energy source, has assumed a renewed importance. Under its Ten Year Plan the NCB aims to boost UK coal production by 15% over the next decade. The US plans to expand its coal industry by a factor of three. Elsewhere—in Europe, the Soviet Union and Australia—similar production drives are getting under way.

But bringing coal out of the ground is one thing; how best to use it is another. The value of coal lies not only in its potential as a fuel. It also shows massive promise as a chemical feedstock, and as a source of premium grade hydrocarbons. Much research is needed—and it is here that NCB (IEA Services) comes in. Set up by the NCB last November, it will look after coal research in Britain for the IEA. Five projects, costing an overall £15 million, have already come its way. Four of them are relatively modest: costing an annual £1 million or so, they involve merely the collection of information for the next five years, keeping the ten nations involved abreast of economic and technological developments.

The fifth project is by far the largest and most promising. It involves the design, construction and operation of an 85 MW fluidised bed combustor on an old NCB site at Grimethorpe in Yorkshire. Jointly financed by Britain, the USA and West Germany, the combustor is expected to cost £6 million to build, and a little over £500,000 a year to run during its eight-year life as an IEA test bed—a total of around £10.5 million.

With so much attention being focused in Britain on nuclear power and North Sea oil resources, the true significance of Grimethorpe is likely to pass unnoticed. In the past, fluidised combustion—an elegant technique for burning fuels efficiently, economically and cleanly—has been sadly under-supported in relation to its technological potential. But Grimethorpe marks a turning point. Through the Depart-

ment of Energy (DEN), which will provide 66% of the cost of Britain's share of the project (the rest will come from the NCB), the UK Government is providing solid backing for the first time.

There are, moreover, preliminary discussions under way between the NCB and leading industrial companies on the early introduction of a 60 MW commercial, prototype in addition to the Grimethorpe test bed. Such a step should be technologically possible, and might have been taken already had funds been available. As it is, success in the present talks, and subsequent government approval, could see the first British prototype operating within five years.

Bed of ashes

Fluidised combustors boast advantages that cry out for commercial exploitation. Because they are more compact and efficient than conventional burners, capital construction costs and running costs are lower. They operate at lower temperatures, forming less clinker and slag and thus lowering maintenance costs. They offer operational flexibility: multifuelled combustors can be designed to burn coal, oil and gas, with switchover times measured only in hours. Thus, while they might usefully exploit fluctuations in different fuel prices, they can also remain immune to temporary shortages of particular fuel types. Moreover, by burning extremely low grade coals they could help stretch economic reserves, reducing overall coal production costs.

These benefits spring from the way fluidised combustion works. The underlying principle is startlingly simple. In a fluidised combustor the proportional amount of air involved in fuel burning far exceeds that found in conventional burners. During combustion, tiny fuel particles are continually fed into a bed of fine mineral ash, maintaining a proportion of 1 part fuel to around 200 parts ash. At the same time, an even flow of air is passed upwards through the fuel bed, causing it to act like a turbulent fluid—hence the name fluidised combustion.

As the fuel particles mill around in the airstream they do not stick together because of the low combustion temperature. Consequently they burn extremely thoroughly, distributing heat more efficiently than under ordinary burning conditions. The heat can be utilised either by passing cooling tubes

through the fuel bed, or by using more air than is needed to maintain turbulence, and extracting it from the top of the combustion chamber.

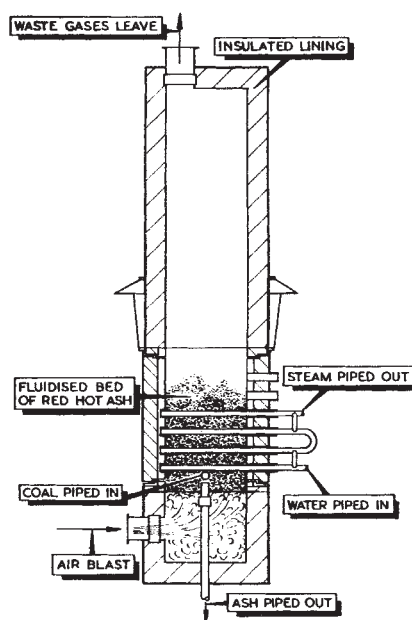
The potential range of application is enormous. As electrical power generators, fluidised combustors might usefully be considered by the Central Electricity Generating Board (CEGB), now busily closing dozens of small, urban coal- and oil-fired plants that could perhaps be converted to complement the nuclear baseload. In industry, fluidised combustors can be used to run small boilers and drying kilns, and pressurised plant can drive gas turbines. They can also be designed as incinerators for sewage sludge, organic and chemical wastes, and everyday municipal rubbish.

With every application the waste heat is easily harnessed. District heating is an obvious area for development here. The DEN, now devoting greater attention to the potential of combined heat and power schemes, is unlikely to overlook this bonus.

The environmental advantages will not be overlooked either. In fluidised combustion the production of SO₂ and nitrogen oxides is easily controlled and minimised. This is because burning temperatures are in the relatively low range of 750–950 °C. On the one hand, this is too cool for the production of most oxides of nitrogen; on the other, it is perfect for the harmless combination of SO₂ with ground limestone placed in the ash bed.

Dusty record

Against that background the poor record of support for research and development (R&D) seems surprising. The CEGB, with potentially so much to gain, withdrew almost entirely from the



Fluidised bed combustor

scene soon after launching pioneering work in the middle 1950s, turning instead to the greater promise then offered by nuclear power and oil. It has since remained involved only in an advisory capacity. Throughout the 1960s the NCB carried the R&D effort virtually alone. The UK Government did not become directly involved until about five years ago, and then only in a minor way with the formation of Combustion Systems Limited (CSL), a tripartite research organisation involving the National Research Development Corporation, the NCB and British Petroleum (BP). The private sector has since shown increasing interest.

The Grimethorpe test bed is not the first in Britain. Progress made by the NCB during the 1960s led to the construction and successful operation of several small experimental rigs at the British Coal Utilisation Research Association (BCURA) in Surrey, and the Coal Research Establishment (CRE) in Gloucestershire. The BP Research Centre at Sunbury on Thames has also been the centre of recent applied research by CSL. Two small commercial plants are already operating in Britain, one of them at Renfrew in Scotland, where the engineering company Babcock and Wilcox runs a 13.5 MW fluidised combustion boiler. The other is used as a grass dryer by an enterprising Lancashire farming family.

Grimethorpe will not represent the first attempt at international collaboration on fluidisation combustion. Work at BCURA in 1970 and 1971 on environmental benefits was sponsored jointly by the NCB and the US Environmental Protection Agency. Over the past five years, following the withdrawal of NCB funding for BCURA, studies on pressurised plant have been supported entirely by the US Office of Coal Research. CSL facilities are currently used by the US Electrical Power Research Institute and the National Swedish Environment Protection Agency, together with many independent commercial organisations. CSL projects are themselves partially supported by grants from the European Coal and Steel Community. With more effort at both national and international level, development might have passed beyond its present point, but the funding was not available.

Perhaps the greatest single setback came in 1971 when the NCB, having decided to stop carrying the R&D burden on its own, failed to win government backing for a planned 20 MW demonstration steam boiler. Only £2 million was involved, and the Advisory Council on Research and Development (then under the Ministry of Power) had just reported favourably on fluidised combustion; initial design studies for the project had been sup-

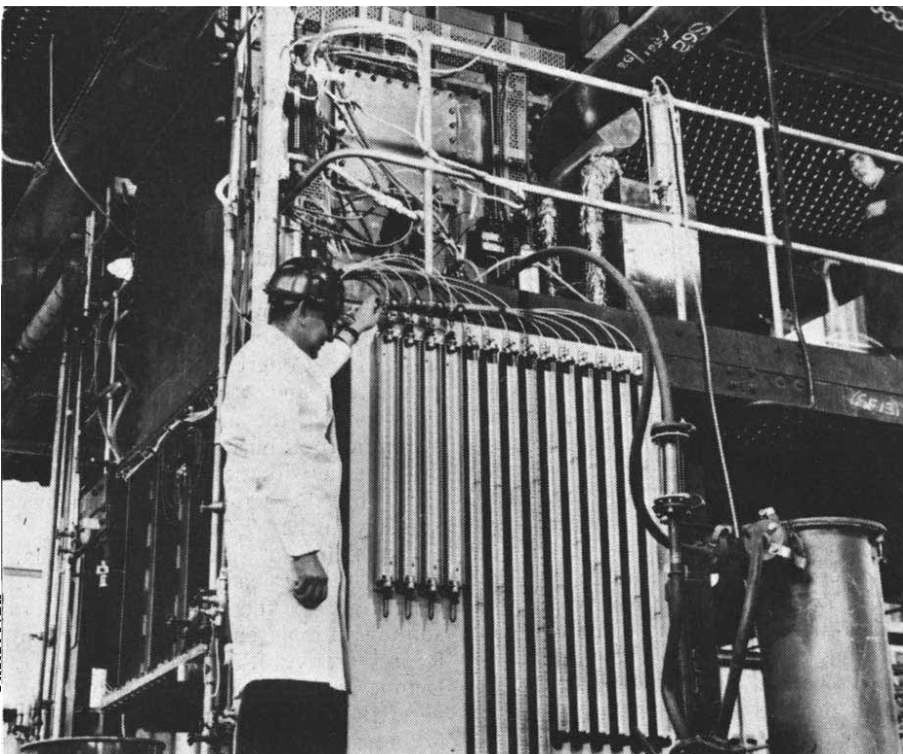


Photo: NCB

Experimental fluidised bed combustor at Coal Research Establishment

ported by government grants totalling £200,000.

But the Tory government had only recently come to power, the CEBG, riding the tide of nuclear expansion, was indifferent about the idea and had itself declined to give support, and even the NCB commitment was less than total. Furthermore, export prospects did not look rosy: the US administration for the most part still spurned fluidised combustion, ironically preferring to place massive emphasis on the development of pollution control units for conventional burners.

Dead ahead

Following that setback it was arguably only through the formation of CSL that Britain maintained its technological lead. Not until the coal industry examination of 1974—involving the government, the NCB and mining unions—did fluidised combustion become identified as a priority area for R&D, along with pyrolysis and liquefaction. The decision to support Grimethorpe with DEN funds acknowledged that finding. Orders for hardware should go to UK, US or West German contractors within the next 12 months.

So fluidised combustion looks set to come into its own. In the UK, apart from Grimethorpe and the planned prototype, CSI is actively considering a 66 MW combined heat and power pressurised gas turbine. The British Steel Corporation may well introduce a small unit into its River Dunn works in Yorkshire. The General Electric Company has declared an interest in the

proposed British prototype. And a new joint company, Babcock-CSL has been established as a design and export base.

In the US too, R&D is pressing ahead independently of the IEA project. Work on a \$15 million pressurised test bed is due to start near Chicago this autumn, sponsored by the US Energy Research and Development Administration. A lot is at stake. While coal makes up 90% of recoverable fossil fuel reserves in the US, much of it has an extremely high sulphur content. Fluidised combustion offers the opportunity to exploit these reserves without violating anti-pollution standards.

The enthusiasm is matched in Europe, in spirit if not with financial backing. Although only West Germany will share the running costs of Grimethorpe with Britain and the US, the other seven member nations of the IEA Working Party on Coal Technology will have access to data. France, alone among the major European nations, has remained cool, but her absence poses no problems.

Fluidised combustion could have tremendous relevance to the future world energy scene. Few now question the crucial importance of nuclear power. And few will argue with the NCB's suggestion that coal's greatest potential lies in pyrolysis and liquefaction. But where coal must be used as a fuel, fluidised combustion looks like becoming the cheapest, cleanest, and most efficient way of using it. As one NCB board member has put it, the greatest innovations are yet to come. □

US ENERGY

Making oil out of money?

A controversial plan to pour billions of dollars of federal funds into commercial development of "synthetic" oil and gas from coal and from oil shale is about to face a crucial test in the US House of Representatives. Colin Norman reports

A SYNTHETIC fuels industry is a key-stone of the Ford administration's long range energy research and development (R&D) strategy. The idea is to force-feed its growth in the United States to the point where it could begin to supplement dwindling domestic supplies of oil and gas in the 1980s and 1990s. The plan now arousing controversy is embodied in a bill already approved in differing versions by the Senate and by four separate House committees. It would provide up to \$6,000 million to private industry over the next two years in loan guarantees and other financial assistance for the design and construction of large scale production plants.

According to the bill's supporters, chief of whom is Vice President Nelson Rockefeller, the measure is vitally needed to halt the growing imbalance between supply and demand from domestic oil and gas. Without the production of synthetic fuels, the argument runs, imports will inevitably increase as domestic supplies begin to peter out towards the end of the century. And without federal support now, there will be no synthetic fuels industry in place when it is needed.

To the plan's many opponents, however, the idea is a waste of money, an unnecessary subsidy to the already bloated oil industry, and a gross misapplication of energy R&D funds.

The latest five-year R&D plan of the Energy Research and Development Administration (ERDA), published last April, envisages a major role for synthetic fuels in the 1990s, suggesting that they "offer a domestic energy alternative to imported oil and gas". ERDA noted, however, that it will require production of between five and ten million barrels of synthetic fuel a day by 1995 simply to hold imports at their present level, and argued that "for the necessary number of plants to be operating in the mid-1990s, an industrial base on the order of 1 million barrels per day must have to exist by 1985".

ERDA's strategy is thus to press ahead with R&D on advanced technology for producing oil and gas from coal and shale, but at the same time it is urging industry to move rapidly ahead with the design and construction

of demonstration plants using technology already developed. The problem, however, is that such large scale plants are expected to cost about \$1,000 million apiece, and, since the economics of the enterprise are difficult to predict, industry is having a tough time raising that sort of money. Hence the argument for Congress to provide loan guarantees and other financial incentives to help the industry get into the synthetic fuels business. ERDA officials describe the situation as similar to that faced by the nascent nuclear power industry in the 1950s.

Opponents of the bill have challenged its justification with arguments which go to the root of the Ford administration's energy policy. Led by Representative Richard Ottinger, a Democrat from New York, they support ERDA's programme for developing advanced synthetic fuel technology—ERDA is already funding a number of pilot scale projects jointly with industry—but they argue that it is premature for the Federal Government to pour vast sums of money into commercialisation efforts.

Those arguments received valuable support late last month from the General Accounting Office (GAO), an investigative body controlled by Congress. In unusually blunt language, the report recommended that "Government financial assistance for commercial development of synthetic fuels should not be provided at this time", and suggested that conservation efforts are likely to be much more cost effective.

The GAO study suggested that oil produced from coal or shale could cost between \$18 and \$30 per barrel, compared with the current price of about \$12 for imported crude. It also estimated that synthetic gas would cost at least twice as much to produce as natural gas. Thus, to make the products competitive, the Federal Government would have to provide direct subsidies in the form of price supports or production assistance, the report suggests.

The GAO report was greeted with jubilation by the bill's opponents, coming as it did on the eve of the House debate. But ERDA officials were understandably less enthusiastic. For example, Robert C. Seamans Jr, the Administrator of ERDA, said in a statement that the report "presents strong conclusions and recommendations to the Congress without a sound underlying basis of analysis supporting them". He took issue with most of the report's conclusions, particularly its

comparison with future synthetic oil prices with present import prices, and suggested, finally, that its publication would be "damaging to the early implementation of our needed energy supply programs".

Equally disturbed was Representative Olin Teague, Chairman of the House Committee on Science and Technology and the chief architect of the bill. Teague called a committee meeting to discuss the report last week, and suggested that he would not bring the bill to the floor unless he is sure that he has sufficient votes to get it through. Later in the week, he said that he would try to get the bill before the full House as soon as possible, indicating that he feels it will pass.

As for the impact of the bill on the synthetic fuels programme, many officials see it as crucial. William T. McCormick, Chief of ERDA's Office of Commercialisation, said last week, for example, that "if this bill doesn't go through, many projects which have been supported by company funds will fold". He added that if the bill fails to pass this year, it will be very difficult to resurrect it next year.

Be that as it may, at least one synthetic fuels programme—the oil shale development effort—is already ailing so much that it may be difficult to revive it simply with a financial injection.

It should be recalled that not too long ago, the vast amounts of oil locked into shale beneath the Rocky Mountains in Colorado, Utah and Wyoming were being touted as the answer to America's energy problems. In January, 1974, the Department of the Interior began to offer small tracts of federal land to the oil industry for testing and development projects. The oil industry paid handsomely for the opportunity, the first 5,000 acre tract fetching a staggering \$210 million.

Last month, however, consortia which hold rights to the tracts now leased in Colorado and Utah, withdrew from the programme and asked that the leases be suspended. And late last year, a consortium known as The Oil Shale Company (TOSCO), which was reckoned to be the leader in shale processing technology, announced that it had suspended its own private operations. They all cited the high capital cost of building the plants and a variety of environmental problems as reasons for pulling out.

If Congress passes the Synthetic Fuels Loan Guarantee Bill it may at least help the economics of shale processing, since it would reduce the cost of borrowing funds on the capital market. But, according to an Interior Department official, the prospects for reviving the effort look gloomy. □

GENETICS

Laying the guidelines bare

The long, tortuous process of setting controls on experiments involving recombinant DNA in the United States has taken yet another potentially important turn. Colin Norman reports

THE National Institutes of Health (NIH), the agency responsible for supporting most recombinant DNA experiments in the United States, last week published a 100-page document analysing the possible effects of the safety guidelines which it issued last June. Publication of the document, a legal requirement under the terms of the National Environmental Policy Act (NEPA), is likely to provide an important means of gathering public opinion on the guidelines, but it could also pave the way for some legal challenges to them.

Links between recombinant DNA research and environmental policy may seem a bit tenuous, but the language of NEPA is clear. It specifies that government actions which could have environmental implications should be preceded by an impact statement detailing the environmental risks and benefits associated with that action. Since microorganisms bearing recombinant DNA molecules could affect the health of plants, animals or man, the issuing of guidelines designed to keep such organisms in the laboratory and out of the general environment clearly comes under the terms of NEPA.

That fact was realised in the NIH while the guidelines were in the late stages of drafting, and caused considerable consternation in the agency, contributing to some delay in publication of the guidelines. In the end, however, Donald S. Fredrickson, Director of the NIH, decided to short-circuit the normal NEPA procedure by publishing the guidelines first and the environmental impact statement later. Therein lies a potential problem for NIH.

Already, NIH has received a number of complaints that Fredrickson's action has circumvented public participation in the formulation of the guidelines. Among the complaints is a letter from two researchers at the Friends of the Earth, a national environmental organisation. The researchers, Francine Simring and Larna Saltzman, have asked NIH to stop funding all recombinant DNA experiments until the correct NEPA procedure has been followed, arguing that prior publication of the guidelines violates the spirit as well as the letter of NEPA.

Fredrickson has claimed, however, that prompt publication of the guidelines served the public interest better than following the usual NEPA procedure. Until the NIH guidelines were published on June 23, recombinant DNA experiments were proceeding under general guidelines drafted by an international group of geneticists which met at Asilomar, California, early in 1975. The NIH guidelines are in many respects more stringent than the Asilomar guidelines and they also establish a detailed implementation procedure. Thus, the impact statement itself suggests that "the escape of potentially hazardous organisms was more likely in the absence of NIH action". Though such arguments are rational enough, the possibility of a legal challenge should not be completely ruled out.

Nevertheless, the impact statement provides an important addition to the literature on the regulation of recombinant DNA experiments since it provides the first public discussion of the reasons why NIH chose not to follow some courses of action advocated by various groups and individuals. It also contains a discussion of the possible risks and benefits associated with the research, attempts to estimate the probability that some hazards will be realised, describes the guidelines, and outlines the procedure by which they were adopted.

As far as alternative courses of action are concerned, the statement discusses the following:

- No action—simply continue to rely on the Asilomar guidelines as the basis for regulating recombinant DNA experiments. Such a course was rejected because the Asilomar guidelines are considered too lax in some cases, and because there is no formal mechanism to ensure that they will be followed.
- Prohibition of funding from NIH for recombinant DNA experiments. That suggestion was rejected because other sources of funding are available, the potential benefits from the research would be foreclosed, it would "undermine American leadership in the establishment of worldwide safety standards", and the "leadership of the United States in biological research would be threatened". A flat prohibition would also interfere with free scientific inquiry, the statement suggests.
- Restrict all permissible experiments to a few maximum containment facilities—a suggestion put forward most notably by Robert Sinsheimer, Chairman of the Department of Biology at California Institute of Technology. The statement notes that such a course of action would not distinguish between experiments with high potential risk and those believed to pose little, if any, hazard. It would also discourage many experiments and amount to a prohibition on some research because of limited access to such facilities, the statement suggests.

● Prohibit the use of the bacterium *E. coli* in recombinant DNA experiments. Because *E. coli* is a common inhabitant of the human gut, many observers have argued that it is a particularly risky choice as the host for transplanted genes. The statement points out that the particular strain of *E. coli* used in the laboratory has many features which limit its infectiousness. Moreover, it can be genetically altered to reduce its viability even further, as required by the guidelines for some classes of experiments. The statement also defends the decision to permit the use of *E. coli* by noting that no other bacterial species now seems to offer as many safety features.

● The statement also notes that some observers have questioned the decision to permit experiments involving the formation of recombinant molecules from uncharacterised pieces of DNA from warm-blooded animals—so-called shotgun experiments. It also notes that the use of DNA from oncogenic viruses has been challenged. The statement does not defend the decision to permit such experiments, however; instead, it merely notes the objections.

● The environmental impact statement has now been circulated in draft form to numerous scientists and other interested individuals and it is also available on request from the NIH division of environmental services. It is open for comments for 45 days, after which the NIH may convene a series of public hearings. Eventually, the document will be expanded and cast in final form. The comments will also be considered during any future revision of the guidelines.

Because both the risks and the benefits from recombinant DNA experiments are speculative, the impact statement is necessarily vague in places, and is also shot through with value judgments in areas where experimental data are lacking. Critics of the guideline and of the research will therefore find plenty to criticise in the document. A heated and lengthy debate should be anticipated. □

IN BRIEF

NRPB evidence published

The UK National Radiological Protection Board (NRPB), which advises the government on radiation hazards, has urged a fresh appraisal of Britain's approach to nuclear waste management. The board's evidence to the Royal Commission on Environmental Pollution (RCEP), disclosed last week, adds credence to the view that the RCEP's forthcoming report on radiological safety in Britain will itself criticise the relatively disappointing level of UK research into the safe disposal of long-lived radioactive nuclides arising from the nuclear power programme.

The NRPB evidence nonetheless questions recent attacks on international exposure limits for plutonium; and it endorses calls for individually established standards for radioactive discharge from nuclear installations, rather than blanket legislation.

AGR running smoothly

Barring unforeseen setbacks the commercial output of Britain's first Advanced Gas Cooled Reactor (AGR) nuclear power station, Hinkley Point B in Somerset, will have doubled to 1,000 MW by the coming winter. The second of Hinkley B's twin 660 MW reactors, now undergoing final engineering tests, is expected to come on-line in October, closely on schedule. The first has been performing smoothly at around 500 MW since July, following its switch on eight months ago—outstanding corrosion problems have restricted output to about 80% of the design level. Hinkley B is already producing electricity as cheaply as first generation, magnox nuclear installations, and could eventually become more competitive.

Though the Central Electricity Generating Board last week expressed renewed confidence in the AGR, which it regards as among the safest and 'cleanest' of existing nuclear reactor

types, export prospects remain bleak because of its comparatively high capital cost and unproven commercial record.

Uranium fix?

Officials from California's State Energy Commission and Public Utilities Commission have in the past few weeks given the US Department of Justice and the Senate Foreign Relations Committee copies of documents obtained by the Australian Friends of the Earth (and published in the Australian press) apparently revealing the existence since 1971 of an international cartel to fix the price of free world uranium.

The documents, apparently obtained from the files of Mary Kathleen Uranium of Australia, are said to contain references to a meeting in Johannesburg in January 1974 attended by representatives from Australia, South Africa, Canada, France and the London-based Rio Tinto Zinc.

No official comment on the matter was available last week from the Justice Department in Washington, which in July served subpoenas on US and Canadian companies in connection with its investigations of a price ring, or from the Uranium Institute in London, which the documents reportedly suggest might have been intended as the successor to the Uranium Marketing Research Organisation, the name purportedly used by members of the "club" to describe their group.

Solar energy gathering

The planning and coordination of the international effort to develop solar energy was one of the subjects which about 150 scientists from more than 50 countries discussed last week when the World Meteorological Organisation (WMO) and UNESCO held a symposium in Geneva. The symposium, a sequel to one held in 1973 at UNESCO headquarters, also discussed problems, both technical and educational, asso-

ciated with the conversion of solar radiation.

Nuclear exports overseer

Following the first meeting of the new French Government, the Elysee Palace last week announced the formation of a high-level government council to oversee France's participation in the growing international trade in nuclear technology. It will "define and co-ordinate sales of nuclear techniques and products".

President Valéry Giscard d'Estaing will head the new committee, which will also include the new Prime Minister, M Raymond Barre, the Foreign, Defence, Finance, Industry and Foreign Trade ministers, and the president of the French Atomic Energy Commission (CEA).

The creation of the new body comes after the call from non-aligned states at their recent conference in Colombo for an oil embargo against France. It also follows the row between France and the US over the sale of a reprocessing plant to Pakistan and the controversy over France's deal involving nuclear plants for South Africa.

Dr Henry Kissinger is meanwhile due to see President Giscard d'Estaing in Paris this week for talks which are expected to include discussions on French nuclear policy.

Biblis to restart

The 1,200 MW Biblis nuclear power station near Essen, West Germany, which was shut down in April after a routine check revealed cracks in the feed-water container, was expected to be back in operation this week, according to a spokesman for the operating utility RWE. News of the fault first emerged in early July, when RWE and Kraftwerk Union, which built the plant, disclosed the cause of the delay. Kraftwerk Union handed the twin pressurised water reactors over in February last year.

Competition 9 Even a common word like electricity was new once. For a prize of £10 we want to hear ideas and terse definitions for new words which will describe some existing or future scientific concept or phenomenon. An example might be 'polyfood'—a food made from, or containing, polyunsaturated fats. The closing date for entries is October 19.

Competition 8 asked for a one-sentence slogan for a laboratory,



business or university. The high quality of the entry forced a sharing of the £10 prize between Simon Bright of Hertfordshire, UK, whose slogan

for British Thornton (or any other manufacturer of slide rules) was "Slide Rules OK", and Alan Mellors and David Piggins of the University of Guelph, Canada, who for the Porton Down Bacteriological Research Establishment offered the slogan "Our enthusiasm is infectious". F. A. Smith of the University of Adelaide, Australia, receives honorable mention with his suggestion for a Forestry Department: "We saw the trees for the wood".

news and views

Harvesting whale and fish populations

from Robert M. May

FROM August 31 to September 9, the Advisory Committee on Marine Resources of the UN Food and Agriculture Organization is holding a Scientific Consultation on Marine Mammals in Bergen. The background papers for the meeting make a fair-sized pile. The major findings are summarised in the reports of four *ad hoc* groups, focusing on large cetaceans, on small cetaceans and sirenians, on seals and marine otters, and on ecological and general problems. A recurring theme is the difficulty of obtaining reliable estimates of population density, and of transferring estimates of population density in one area to another. For example, in reviewing population assessments of Antarctic fin whales, Gambel (conference document ACMRR/MM/EC/9) observes that the International Whaling Commission (IWC) has agreed on a current population estimate of about 83,000 for this species. Gambel notes that this estimate rests, *inter alia*, on a particular model, but that both the model and the numerical estimates of its key parameters are open to question. Moreover there appear to have been "sharp but erratic declines in the fin whale stock indices" in the areas designated III and IV by the IWC, and also a sharp decline in the catch-per-unit-effort in area V; these declines are not consistent with the IWC model. This erratic behaviour is typical of many exploited whale and fish populations, where the amplitude of population fluctuations increases as harvesting rates increase.

In theory and in practice, a central concept in the harvesting of a natural population is the maximum sustained yield, or MSY. The population may be thought of as having some intrinsic nett growth rate, dN/dt , which depends on the population density, N : at low densities, the per capita growth rate is relatively high, but the population is itself small, so that initially the overall growth rate increases as N increases; at high densities, the per capita growth rate typically falls, attaining zero when N reaches a value K (the "carrying capacity" of the environment), and

then becoming negative. Increases in per capita fecundity as population levels are lowered have been documented for several fish and marine mammal populations, although the underlying mechanisms are not always well understood. Possible explanations include the greater wealth of resources in a less crowded environment, and a variety of behavioural responses (some of which, in gregarious marine mammals, can stem from the breakdown in social organisation attendant upon the removal of dominant males or females). The upshot is that the typical plot of total population growth as a function of population density (dN/dt against N) rises from zero at $N=0$, attains some maximum value at $N=M$, and then declines back toward zero at $N=K$, thereafter remaining negative. A familiar equation which represents this phenomenon is the logistic $dN/dt=rN(1-N/K)$, but this is only one of many equivalent forms (see for example Holt, ACMRR/MM/EC/29).

In its natural, unharvested state the population will be around the equilibrium value, where recruitment balances mortality, at $N=K$. Harvesting constitutes an additional source of mortality, and (provided the harvesting rate is not too high) the population will decrease to some new steady value at which the population gains from the intrinsic growth rate exactly balance the losses due to harvesting. The MSY is achieved by harvesting at a rate such that the population remains around $N=M$, that is, by harvesting at a rate equal to the maximum possible nett growth rate. The population may be thought of as a capital sum in a bank, with however the odd property that the interest rate depends on the magnitude of the sum. For sustained yield, one harvests only the interest on the capital; and for maximum sustained yield one arranges that the capital sum shall be that which generates the maximum interest rate.

Two papers in the collection call attention to shortcomings in overly simple notions of MSY, and make important extensions to the theory.

Holt, the convenor of this con-

ference, gives a catalogue of different formulae which have been used to describe recruitment curves. He shows that, on the one hand, available data are rarely of sufficient accuracy to discriminate among the alternative forms, but that, on the other hand, the different forms can lead to significantly different predictions as to MSY. In extreme cases, the harvesting rate that gives the MSY for one curve (for example, a curve skewed to low densities, such as the Beverton and Holt $dN/dt=(1-b)(1-N)N/[N+(1-b)N]$ with $b=0.33$) can result in extinction of the population if some other curve in fact pertains (for example a curve skewed to high densities, such as the Pella and Tomlinson $dN/dt=bN(1-N^2)$ with $b=0.67$). Holt thus cautions against the uncritical use of MSY calculations to set quotas (see ACMRR/MM/SC/61, and also Allen, SC/57, and Gulland, SC/82). He recommends that a range of different quotas should be set for different stocks of the same species (in different geographical regions), these quotas being set on the basis of a spectrum of estimates of the state of the stock and the form of its recruitment curve; in this way we may hope to learn more.

Holt and the *ad hoc* group IV (ACMRR/MM/SC/5) also note the need to incorporate the effects of random environmental variability into simple population models. One outcome of preliminary explorations in this direction (Beddington, personal communication) is the suggestion that as harvesting rates increase, so too do the relative fluctuations in the population magnitude, and in the catch itself. This goes some way toward explaining an observed property of exploited populations, which was noted above.

Clark (conference document ACMRR/MM/SC/65, and also *Science*, **181**, 630-634; 1973) makes the important observation that the aim of most commercial fish and whaling industries is not so much to maximise sustained yield, as to maximise the present value, PV, of (discounted) nett economic revenue. To this end, Clark takes the sort of considerations used

for MSY calculations, and modifies them in two ways. First, the harvesting costs are subtracted from the harvest, itself expressed in money units. Second, the present value of the nett economic revenue is obtained by integrating over all future yields, discounting at some appropriate discount rate, δ (which will usually be of the order of the bank interest rate, $\delta=10-20\%$). The aim is now to maximise PV. Although this approach is oversimplified in many ways (it neglects fixed capital costs for example), it is more realistic than classical MSY calculations in that it automatically incorporates the trade-off between short-term and long-term benefits.

Clark then shows that PV is maximised by a strategy which initially depletes the stock to some predetermined value, L , and thereafter harvests it for sustained yield (keeping $N=L$). His remarks are illustrated by numerical calculations for particular assumptions on recruitment and cost curves, but the conclusions are clearly general. Unless we have a combination of relatively low discount rate and relatively high harvesting cost, L will be less than the classical MSY point at $N=M$. The MSY approach will, however, give a good first approximation so long as the discount rate δ is significantly less than the intrinsic per capita rate of population growth, r , for the harvested species. Conversely, if δ is greater than, or of the order of, r then the sustained population value for maximum PV is significantly smaller than the MSY value; $L \leq M$. Clark observes that "international institutions such as the IWC seem to have been established on the assumption that the economic interests of the industry would, if properly channelled, automatically ensure the conservation of the resource". He emphasises that his "analysis indicates that the economic incentive for conservation of such resources may be quite minimal, as far as the commercial industry is concerned. The economic values of the whaling industry, of course, may not coincide with long term economic values of society as a whole".

If, for the moment, harvesting costs are neglected, then a simple but rigorous generalisation of Clarke's results can be given: maximisation of the present value of discounted nett economic revenue of a harvested population will permit the population to persist if, and only if, the maximum per capita population growth rate exceeds the discount rate. If not, PV is maximised by harvesting to extinction. In particular, if the overall population growth curve is logistic, the ratio of maximum PV to MSY sustained populations is $L/M=1-\delta/r$ if $\delta < r$, and zero if $\delta > r$. Returning to the



American Indians whaling in the 17th century

image of a fish population as a capital sum in a bank, we see that if the fish are not capable of an intrinsic per capita growth rate in excess of the bank interest rate, then we do better to turn the entire capital sum—the entire fish stock—into real money in a real bank.

For many commercial fisheries in the North Sea, r is indeed sufficiently large for conventional MSY calculations to retain validity as sensible first approximations. But for the Antarctic blue whale r has been estimated at around 5%, which as Clark emphasises is less than (or at least well within) the normal range of discount rates. Consequently PV may be maximised by fishing such whale species virtually to extinction. The policies pursued by the whaling industries of countries like Japan are not the result of brutal stupidity, but rather are the optimal solutions of problems defined in narrowly economic terms.

Returning to the question of harvesting costs, we note that these will typically be higher per unit catch at lower population densities. Thus it is never economically rational to harvest the last few animals. There remains the worry that this lower economic breakpoint may be below some biological threshold population density, where for geographical or behavioural reasons the population is incapable of maintaining itself; ecologists refer to the existence of such a threshold as an "Allee effect". No such Allee effect has been demonstrated for any marine mammal, so that this economic consideration may contain one gleam of cheer.

Clark's work, which is developed further in his book *Mathematical Bioeconomics* (Wiley, New York, 1976),

is very illuminating. It undercuts the pious belief that the interests of conservation and industry can march together if only the world is seen clearly. The conservationist is essentially a person who does not discount the future, a person for whom $\delta=0$; for the industrialist, δ is typically the prevailing bank interest rate. Their interests can thus be fundamentally irreconcilable, and no amount of methodological sophistication can help. The overriding decisions must be qualitative social and political ones, on the effective rate at which we discount the future of the world. Then, and only then, can we usefully employ the technical apparatus of MSY, optimum present value of discounted economic revenue, and the like. □

Polycyclic aromatic carcinogenesis

from S. Neidle

OF all the many external agents which can induce cancerous growth in animal cells, polycyclic aromatic hydrocarbons have been the most intensively studied, and indeed this topic has for long occupied a central position in cancer research. Just 200 years ago Pott first described the effects of soot and coal tar in producing a high incidence of scrotal cancer in chimney sweeps—it was not until the 1930s that Kennaway and Cook isolated in pure form the active chemical carcinogen from coal tar and identified it as benzo[*a*]pyrene, a polycyclic aromatic molecule with five condensed six-

membered rings, and an almost planar molecular geometry. Since then a large number of analogues both potent and inactive have been discovered and/or synthesised.

In spite of considerable research over the past two decades, the molecular basis of action of these compounds is still imperfectly understood, however. An initial problem still without a definitive answer concerns the cellular target of these carcinogens. Both proteins and nucleic acids have been implicated by various workers; however it is now generally agreed that DNA is probably the most likely candidate. Certainly, binding of carcinogenic polycyclics to DNA, albeit at exceedingly low levels, has been well established, largely through the extensive studies of Brookes, Sims and their collaborators. At first sight, it is attractive to conceive of this binding in terms of intercalation between DNA base pairs, as occurs with many planar drug molecules, especially since the hydrocarbons themselves are chemically unreactive. However, there is now clear evidence that the binding is covalent both *in vivo* and *in vitro*. Moreover, intercalation presupposes a planar molecular conformation for the substrate, and there are several examples of very active polycyclic carcinogens which are markedly non-planar and therefore could not possibly act as intercalators, as Glusker, Zacharias and Carrell have shown by X-ray crystallography in the case of 7-chloromethylbenz [a] anthracene (*Cancer Res.*, **36**, 2428; 1976). An alternative hypothesis by Boyland that binding involves an intermediate metabolite, such as an epoxide, has in recent years received widespread general acceptance, and it is now believed that the microsomal "mixed-function" oxidase system is responsible for this metabolic activation into a chemically reactive form.

A region of the polycyclic hydrocarbon molecule considered to be of likely accessibility to such activation is the so-called K-region. Indeed, Grover and Sims have shown that K-region epoxides do bind to DNA both *in vivo* and *in vitro* (*Adv. Cancer Res.*, **18**, 165; 1974), and are more active than the parent hydrocarbons. Weinstein, Harvey *et al.* have investigated the *in vivo* binding of such an epoxide, 7,12-dimethylbenz [a] anthracene 5,6-oxide to various nucleic acids and synthetic homopolynucleotides (*Biochemistry*, **14**, 3451; 1975) and showed that there was a preference for reaction with the guanine bases in poly(G). They have now followed up this work with an elegant characterisation of the four major guanosine adducts obtained from separation of the hydrolysis products from the poly(G)-hydrocarbon complex (*Proc. natn. Acad. Sci. U.S.A.*, **73**,

Sounds volcanic

from Peter J. Smith

ACOUSTIC noise from volcanoes must have been heard for thousands of years; yet surprisingly perhaps, no recordings of volcanic sound appear to have been made before the 1950s (in Japan). The history of the scientific study of volcanic noise is not quite as short as that would imply, however. What were probably the first systematic observations were made at Vesuvius in 1906 by Perret, who later continued his work at Etna, Stromboli, Kilauea, Pelee and elsewhere (see *Volcanological Observations*, Carnegie Inst. Wash. Publ. 549, 1950). More recently, Wilcox (in *US Geol. Surv. Bull.* 965-D, 1956) has described 11 distinct sounds from Paricutin, and Richards (*J. geophys. Res.*, **68**, 919; 1963), in a wider study, has correlated different sounds with the type of volcanic activity producing them.

But there is more to the study of volcanic sound than simply description and classification. Gorshkov (*Bull. Volcanol.*, **23**, 141; 1960), for example, used microbarograph records to estimate the energy discharged to the atmosphere by several large eruptions, the largest of which was the Krakatoa explosion of 1883 (8.6×10^{23} erg). The Shiveluch eruption of 1965 was rather smaller, with only 1.8×10^{21} erg of energy emitted in air waves; but Gorshkov and Dubik (*Bull. Volcanol.*, **34**, 261; 1970) were able to take the analysis of this particular event even further. They found that only 0.10–0.15 per cent of the total kinetic energy derived from the eruption was imparted to the air. According to their estimate, the total energy of eruption was 1.3×10^{25} erg, most of which was transmitted in the ejecta as thermal energy.

A second quantitative line of enquiry derives from the origin of the sound. The acoustic noise asso-

ciated with volcanic eruptions is, of course, due to the flow of gases—in particular to the interaction of gases with the stationary sides of the volcanic vent and to turbulence in the gases themselves. So as Woulff and McGetchin (*Geophys. J.*, **45**, 601; 1976) point out, analysis of volcanic sound should provide information about volcanic gas behaviour in general and about the velocity history of the gas in particular.

For example, a theoretical study carried out by Woulff and McGetchin suggests that three types of acoustic radiation from volcanic gas flow are possible—monopole, dipole and quadrupole. But the question then arises as to which of these occur in practice. Unfortunately, observational data are sparse; but analysis of recordings of the sound associated with fumarole activity at Volcan Acatenango, a stratovolcano in Guatemala, shows that in this particular instance the radiation is predominantly, if not entirely, dipolar. Extrapolation of this result to other types of volcanic activity is difficult, of course, although the little information available suggests that dipolar radiation should also be predominant in the more violent strombolian type of eruption, at least as long as the gas flow is subsonic.

Evidently quantitative studies of volcanic sound are still in their infancy. If acoustic data are to be translated into absolute gas flow velocities it will be necessary to know not only the type of radiation predominant in each particular case but also the constant of proportionality relating velocity to acoustic power in the power law appropriate to that radiation type. Again, the latter information is largely unavailable at present. As an intermediate step, however, there is the hope that even incomplete data will allow the elucidation of relative changes during an eruptive history and thus contribute to an understanding of eruption dynamics.

2311; 1976). In all four adducts, linkage with the polycyclic aromatic is by way of the 2-amino guanine group; two diastereoisomers are linked to the 5-position of the hydrocarbon, and the other two, are similarly linked to the 6-position.

However, there is increasing and convincing evidence that these K-epoxides are not the major carcinogenic intermediates, at least for some hydrocarbons. Thus, Baird *et al.* have found that 7-methylbenz[a]anthracene and its K-epoxide form significantly different RNA-bound products on treatment with mouse embryo cells in culture (*Cancer Res.*, **36**, 2306; 1976), although

they were unable to actually characterise these products. This is of course not surprising, since the extent of *in vivo* binding is low—at most one hydrocarbon molecule per 10^4 nucleotide residues. Recently devised techniques of extremely sensitive spectrofluorimetry have helped in this respect. Thus, Daudel *et al.* (*FEBS Lett.*, **57**, 250; 1975) have been able to compare directly spectra of DNA isolated from solution-binding with benzo[a]pyrene, with those from treated mouse skin as well as with spectra from several putative metabolite-DNA complexes. Their results clearly indicate that the K-region 4,5-epoxide is not the species

that reacts with DNA, and instead provide suggestive, though not conclusive evidence that a non-K-region diol-9,10-epoxide is primarily implicated in the metabolic activation of benzo[a]pyrene. Support for this has also come from the similar studies of Weinstein *et al.* (*Biochem. biophys. Res. Commun.*, **70**, 1172; 1976). Weinstein, Harvey and their collaborators have now succeeded in isolating and identifying the adducts from RNA degradation after *in vivo* incubation of benzo[a]pyrene in bovine bronchial explants. The intermediate is the (1) 7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydro derivative, that is a non-K-region diol epoxide, with subsequent attachment of the 10 position to the 2-amino guanine group (*Science*, **193**, 592; 1976). This compound has also been shown by Newbold and Brookes (*Nature*, **261**, 53; 1976) and Sachs *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, **73**, 607; 1976) to be by far the most active mutagen (and therefore carcinogen) of a range of possible benzo[a]pyrene metabolites, including the K-epoxide.

There still remain however, several unanswered questions concerning the ultimate carcinogenic intermediates arising from benzo[a]pyrene, and indeed from polycyclic aromatic hydrocarbons in general. Certainly the diol epoxide is by no means the only reactive intermediate; others remain to be isolated and defined. One should perhaps add that although there is now some evidence that the benzo[a]pyrene diol epoxide is an intermediate in other tissues and species during hydrocarbon binding to DNA, it is not really known whether the same is true for binding to other cellular macromolecules. $\square$

Nucleic acid modification at Erlangen

from Ernest Borek

An international conference on post-transcriptional modification of nucleic acids, organised by Drs Helga and Walter Kersten, was held at the University of Erlangen on July 19-24, 1976.

THE field of nucleic acid modification is burgeoning, making this a most timely meeting. In eukaryotes every species of nucleic acid is modified and the number of known modifications is increasing—six new modifications were reported at this meeting.

W. Kersten (University of Erlangen)

led a round table discussion on the status of tRNA methyltransferases and isoaccepting tRNAs in tumour tissue. In all tumours, except benign ones, the tRNA methyltransferases are altered both qualitatively and quantitatively compared with their counterparts in normal tissue. All tumours examined contain novel isoaccepting tRNAs some of which are also found in embryonic tissue. There is no evidence for extensive hypermethylation of bulk tRNA isolated from tumour tissue, however, but since there are only a few tumour-specific isoaccepting tRNAs in each tumour, each of these purified tRNAs will have to be analysed. The extraordinarily high turnover of tRNAs in tumour tissue has been established recently by reliable analyses of urinary excretion products stemming from the breakdown of tRNA, determined at the US National Cancer Institute. A normal adult may be excreting about 2 mg of dimethylguanosine in his urine per day whereas some patients with Burkitt's lymphoma and cancer of the breast may be excreting as much as 20 mg. If we assume a tumour burden of 1-2 pounds per 100-pound patient, then 1-2% of his metabolising machinery may be producing the breakdown product at a rate 500% higher than a corresponding

amount of normal tissue. Some unknown effect of the tumour on the metabolism of the rest of the body cannot be excluded. Such a high turnover of tRNA has also been demonstrated recently in an animal model.

The significance of these aberrations in tumour metabolism is, of course, obscure. They may be part of the oncogenic process or they may contribute to the maintenance of the morbid condition. In view of the many functions and activities of tRNA the problem presents an interesting challenge.

Nothing is yet known about methylation of DNA in tumour tissue. The technology of eukaryotic DNA methyltransferase is still lagging behind the rest of the field, because of the impossibility until recently of preparing soluble enzymes, and there are still conflicting reports of cofactor requirements.

Large strides have been made, however, in the understanding of rRNA modification and processing. J. Dahlberg (University of Wisconsin) identified some of the "spacer" polynucleotides between 16S and 23S rRNA genes as tRNA genes, two of which have been identified as tRNA^{Glu} and tRNA^{Ile}. This distribution can account for the coordinate control of the transcription of tRNA and rRNA. R. J. Planta (University of Amsterdam) reported on the species specificity of rRNA modification. The ψ (pseudouridine) content of bacteria, yeast and human rRNA, per thousand nucleotides, is 2.5, 8 and 12 respectively. New modified nucleosides are still turning up. Planta has identified a ψ methylated at N₁ and hypermodified at N₃ by an α -amino- α -carboxyl-propyl moiety. (The latter stems from methionine by the elimination of the thiomethyl group.) Nishimura had reported earlier on the modification of U in tRNA by the same fragment of methionine. The versatility of S-adenosylmethionine as the progenitor of modifications is astonishing.

Nishimura (National Cancer Institute, Tokyo) reported his latest studies on the fascinating hypermodified Q base which is present, adjacent to the anticodon, in several tRNAs of many species. What had been thought to be an isomer, Q*, is actually a still more modified form of it. In Q*, position 4 of the cyclopentene ring can be condensed with either mannose or galactose. The method of achieving modification of tRNA by the Q base is fascinatingly complex. In 1972, Farkas (University of Tennessee) discovered an enzyme which inserts guanine into tRNA^{His} of reticulocytes. Nishimura has now found a function for this "guanine insertase"—it introduces a guanine to replace the Q base

Curious atom

from W. T. Toner

A HYDROGEN-like atom in which short-lived elementary particles substitute for both the nucleus and the orbital electron has been detected as the direct product of the decay of a third unstable elementary particle. Professor Schwartz and his colleagues (Coombes *et al.*, *Phys. Rev. Lett.*, **37**, 249; 1976) hope that a study of this most intriguing scientific curiosity will yield new information on the properties of π and μ mesons.

The parent is the longer lived of the two neutral K-mesons, the K_L⁰ which frequently decays into a π meson, a μ meson and a neutrino. In a minute fraction ($\sim 10^{-7}$) of these decays, the oppositely charged mesons are emitted travelling by chance in the same direction with such similar velocities that they remain together long enough to be bound by their mutual electrical attraction. The bound systems are observed after they have passed down a long straight channel swept clear of charged particles by a magnetic field, in an apparatus which detects their charged components when the " π - μ atoms" are dissociated in an aluminium foil.

without cleavage of the phosphate linkage. Whether an enzyme to reverse the structure, "Q insertase", also exists is not yet known. The complexity of these reactions is significant in view of the known fluctuation in Q base content in *Drosophila* during metamorphosis, and the elevated content of Q* in tumour tissue.

H. Kersten (University of Erlangen) reported on the origin of the methyl groups in thymine of tRNA in Gram-positive organisms. In all but one out of some 20 tested the methyl group is synthesised at the macromolecular level by way of the folate pathway. In all other organisms, including mammals, that methyl group originates from S-adenosylmethionine. On the other hand, the methyl groups in thymine in the rRNA of Gram-positive organisms come from S-adenosylmethionine. It was pointed out that the folate pathway of synthesis must be the more primitive since this is the origin of the methyl group of thymine in DNA. Methylation by enzymes at the macromolecular level is probably a later development since it is the method used to imprint species individuality on DNA and in tRNA. An attempt to resolve the paradox of the two pathways for the methyl groups in tRNA and rRNA was attempted by W. Kersten by the suggestion that tRNA is more primitive than rRNA. This is an interesting suggestion for those interested in the evolution of biomacromolecules.

H. Aschoff and W. Kersten (Erlangen) reported on an 800-fold purification of tRNA 7-G trans-methylase from *E. coli*. Starting with kilos of *E. coli* they obtained a protein of 300,000 daltons which produces only 7-meG in tRNA from *B. subtilis* in which a G in the extra arm is not methylated. This signal achievement 15 years after their discovery of the trans-methylase highlights the difficulty inherent in purifying these complex enzymes.

One session was devoted to the modification of mRNA in eukaryotes. This area, too, is becoming increasingly complex. For example, D. T. Dubin (Rutgers University) has found a hitherto unknown "capping" of Sindbis virus-specific mRNA with an N₂-dimethyl-7-monomethyl G.

It is interesting to note the impact of policies of governance of science on the particular field covered by the meeting. Pre-eminence in the technology of tRNA modification has now passed from America to Germany and Japan, and one reason for this must lie in the pattern of the granting mechanism in the USA since no one in his right mind would dare tackle such problems on a 2-year NSF grant. There have been two international

conferences on nucleic acid modification in the past 2 years; the first one was convened by Italian colleagues and the current one by German workers. To neither of these conferences was a contributor from Great Britain invited, which may well be because the MRC decided a few years ago that the study of modification of nucleic acids should have a low priority for support. What a pity to exclude the people who initiated modern biochemistry from tackling one of the most challenging problems in the field. □

Mysterious meteorites

from M. G. Edmunds

Some forty meteorite chemists and astrophysicists met for an international four-day workshop on isotopic abundance anomalies organised by the Department of Applied Mathematics and Astronomy, University College, Cardiff, and held at Gregynog Hall on August 10-13, 1976.

THE early history of the Solar System has always provided ample scope for speculation, yet recent experimental investigations of the isotopic composition of certain meteorites have provided even more food for thought. A class of meteorites, the carbonaceous chondrites, has traditionally been regarded as the best available sample (except for some volatile elements) of the material out of which the Solar System formed. It came as rather a surprise, therefore, when work in the past few years showed that the relative isotopic abundances of particular elements in these meteorites are anomalous in that they can differ significantly from the so-called "cosmic" abundance ratios which are based on an amalgam of meteoritic, solar spectra and solar wind data. The workshop at Gregynog evolved into discussion of two major topics. The first problem was to sort out the experimental evidence to see exactly which elements really do show isotopic anomalies, and the second was to sift through possible theoretical mechanisms which could account for the origin of these anomalies. The three main possibilities examined were: chemical fractionation of isotopes occurring within some region of the early solar nebula out of which solid planets condensed; the production of particular isotopes in nuclear reactions caused by irradiation of the nebula by

Coated vesicles and clathrin

In a recent squib in these columns, Matus suggested that the protein molecule, "clathrin", which forms the lattice-like coats around coated vesicles (Pearse, *J. molec. Biol.*, **97**, 93; 1975; *Proc. natn. Acad. Sci. U.S.A.*, **73**, 1255; 1976; Crowther *et al.*, *J. molec. Biol.*, **103**, 785; 1976) should be renamed "cytonexin". His reason for this is that E. G. Gray had described a fibrous network in presynaptic terminals which he called a cytonet (*J. Neurocytol.*, **1**, 363; 1972; *Brain Res.*, **62**, 329; 1973). At that time, Gray noted that the bristles on coated vesicles were indistinguishable (in the electron microscope) from the surrounding cytoplasmic matrix. As Matus states, Gray (*J. Neurocytol.*, **4**, 315; 1975) has since repudiated the existence of this cytonet, concluding that it is an artefact of fixation probably arising from the precipitation of cytoplasmic proteins. Despite this, Matus now wishes to preserve this cobweb from the past and make us believe that the cytonet still exists, and that its constituent molecules can be identified by electron microscopy as clathrin, the molecule of which the striking polyhedral coats of coated vesicles are made.

B. M. F. PEARSE

M. S. BRETSCHER

A. I. MATUS REPLIES: Pearse and Bretscher complain about my rehabilitation of the cytonet on the grounds that Gray has repudiated it (*J. Neurocytol.*, **4**, 315; 1975). However in that paper Gray ascribed both the cytonet and vesicular coats to a fixation artefact. If we are now to believe that vesicle coats are a genuine feature of the *in vivo* state, as Pearse's work suggests, then Gray's earlier description of the cytonet (*J. Neurocytol.*, **1**, 363; 1972) is also unchallenged. Having seen many of Professor Gray's micrographs I have never doubted its existence. However, contrary to the suggestion made by Pearse and Bretscher I have no desire to make anyone believe in the cytonet. I do believe that it is important to point out an alternative to the restricted view afforded by their premature neologism for the coat/cytonet protein.

review article

Electrochemical, solid state, photochemical and technological aspects of photoelectrochemical energy converters

Joost Manassen, David Cahen & Gary Hodes*

Avraham Sofer†

Different aspects of photoelectrochemical energy conversion are discussed. It is shown, by conservative estimates of the energy losses in such a system, that the available photopotential is smaller than the optical bandgap of the semiconductor by at least 1 eV. It is shown that water decomposition using only one photosensitive electrode is energetically unfavourable and that, even when two photosensitive electrodes, are used, the production of electrical current is to be preferred over that of hydrogen.

THE idea of converting solar radiation directly into electrical energy by using electrochemical cells, is gaining in popularity¹⁻⁹. It is a multidisciplinary field, which covers electrochemistry, solid state physics and photochemistry as well as technology and economics⁶⁻⁹.

From reviewing the publications in these fields it is clear that specialists in one field often are not aware of the implications and restrictions of another field. It is therefore our intention to discuss in this paper some simple general principles rather than to discuss the different aspects in depth as is done in the specialised texts.

Electrochemical aspects

If an electrode is immersed into an electrolyte solution containing a redox couple R/O and is held at a certain potential, two reactions occur at the electrode surface:

(1) The cathodic reaction $O + ne \rightarrow R$ (e = electron, n = reaction valence) and

(2) The anodic reaction $R \rightarrow ne + O$

If both reactions are rapid and the electrode is held at the equilibrium potential of the couple, a situation is established where the concentrations of R and O are equal. At more anodic potentials, O will be produced, and vice versa. In the case of a slow anodic reaction, for kinetic reasons, however, an anodic overpotential has to be applied to produce O, and the same applies to R when the cathodic reaction is slow. In cases where the cathodic and anodic reactions are one-step processes, we may expect the same kinetic barrier to exist for both reactions and both to be retarded accordingly, but this need not necessarily be so for a multi-stage reaction (see section on energy conversion).

In a photoelectrochemical energy converter, a photoactive electrode is connected with a counter electrode, which should

be as rapid as possible towards the reaction reverse to the one occurring at the photoactive electrode. If the photoactive electrode is illuminated, an electrical current may flow for two quite distinct reasons: one is photocatalysis and the other energy conversion.

Photocatalysis

Photocatalysis can occur only when the system is in a state such that current flow is thermodynamically possible, but is prevented by the kinetics. Such a thermodynamic state may be created, for example, if both electrodes are held at a different potential either by means of an external power source or by keeping anolyte and catholyte at a different composition or temperature. Current can be negligibly small under these thermodynamically favourable conditions, for example because of an overpotential at the photoactive electrode for the redox reaction. The kinetic barrier may be lowered by illumination leading to an increase in current flow. This current flows however because of photocatalysis of an electrochemical reaction and without conversion of light into electrical energy. Another possibility is that the presence in the solution of a material which can react chemically with R or with O, but fails to do so for kinetic reasons. Again, illumination may lower the kinetic barrier and because R or O is consumed, an electrical current will start to flow. In this case we have a photocatalysed fuel cell and again no conversion of light into electrical energy.

Energy conversion

Energy conversion can occur only when illumination changes the thermodynamics of the system, and not just the kinetics as in the case of photocatalysis. In photocatalysis we lower a kinetic barrier and get a decrease of overpotential. In energy conversion we change the energy level of one of the components of the reacting system and must get an underpotential. This means that illumination leads to a situation which in the dark is thermodynamically unfavourable. There is an additional

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Table 1 Currents, potentials and power-output of a TiO_2 photo-electrochemical cell, as a function of the counter electrode

Counter electrode	Short-circuit current (μA)	Open-circuit potential (mV)	Maximum power (μW)
O_2 bubbling through solution			
Platinum on high surface carbon black	640	760	200
Platinised platinum	640	760	200
Smooth platinum	530	670	143
Without bubbling			
Platinum on high surface carbon black	640	760	200
Platinised platinum	580	720	175
Smooth platinum	460	640	120

Electrolyte: 1 N KOH. Light source: 150 W tungsten/iodide. (Intensity equivalent to sunlight incident on the cell.) TiO_2 polycrystalline. Surface: TiO_2 electrode, 0.75 cm^2 ; counter electrode, 4 cm^2 .

condition, however. Imagine that illumination causes an anodic underpotential, which means that O is produced at potentials lower than the equilibrium potential. If the cathodic reaction is rapid, O will be reduced immediately back to R and no current will flow to the counter electrode. The additional condition is, therefore, a slow back reaction at the photoactive electrode.

An attractive system is the redox couple $\text{O}_2/\text{H}_2\text{O}$, which has been much studied in connection with the photoactive TiO_2 electrode^{1-3,5,7-11}. If a TiO_2 electrode is illuminated with light, having an energy larger than its optical bandgap (see below), oxygen is evolved at potentials of more than a volt lower than the thermodynamic oxygen evolution potential. This does not mean however, that in a photoelectrochemical cell with a TiO_2 anode, hydrogen is evolved at the counter electrode. According to the principles described here, oxygen, if present, will be reduced back to water at the counter electrode. Electrodes, which have a low overpotential for oxygen reduction are known from fuel cell technology and in Table 1 (J.M., G.H. and D.C., unpublished results) we show how the performance of such a photoelectrochemical cell (PEC) can be improved by changing the counter electrode.

The slow back reaction is ensured in this system because TiO_2 shows a large overpotential for oxygen reduction.

Hydrogen evolution has only been proven in this system by either giving a cathodic bias^{7,10} or by having catholyte and anolyte of different composition^{7,11}. In both cases a clear distinction has to be made between energy conversion with the assistance of light, and energy conversion solely by light.

In the next section we shall show that to maximise efficiency, overpotentials should be minimised. At the counter electrode this can be achieved by the use of electrocatalysts and a high surface area, as we have seen. At the photoactive electrode we demand a slow back reaction, however, and we have seen that for many cases this means that the forward reaction will also be slow (leading to a sizeable overpotential). An ideal case would be a multistage redox couple, like the $\text{O}_2/\text{H}_2\text{O}$ system, where in principle the forward reaction (oxygen evolution) may be fast and the backreaction (oxygen reduction) may be slow. This, however, is an exceptional case and redox reactions at semiconductor electrodes tend in any case to be slower than at metals. The conditions of a slow back reaction at the photoactive electrode will therefore, in most cases mean that the overpotential of the forward reaction will be appreciable.

Solid state aspects

For an understanding of the origin of the photopotential generated in a photoelectrochemical cell and to estimate its magnitude, it is necessary to understand what is happening in a semiconductor when it is brought into contact with an electrolyte solution and subsequently illuminated. The processes involved can be illustrated schematically by plots of electron energy levels across the semiconductor-electrolyte interface.

Fig. 1a shows the energy levels of an n-type semiconductor in contact with vacuum. When the semiconductor is immersed in an electrolyte solution, containing a redox couple with redox potential E_{redox} , the situation as depicted in Fig. 1b will result, after thermodynamic equilibrium between the two phases is established (that is, the electrochemical potential in both phases is equal). [To put the redox potential on the same energy scale as that used for the semiconductor, both can be referred to the energy level of an electron *in vacuo* at infinity (E_{vac}). It has been shown that $E_o(\text{redox})$ in volts $- E_o(\text{redox})$ in eV $+ 4.5 \text{ eV}$, that is the energy level for the H^+/H_2 redox couple is 4.5 eV below¹² that of E_{vac} (4.3 eV according to ref. 13). We assume here, for simplicity, that surface states do not have a role in determining the energetic picture of the electrolyte-semiconductor interface, that is, we deal with the so-called Schottky case. If they do play a part, they can determine the band bending to a greater or lesser extent, independent of the electron affinity of the semiconductor.] This equilibrium will be reached by the passing of majority carriers (electrons in this case) from the semiconductor to the solution, causing a redistribution of positive and negative charge carriers at and near the surface until the Fermi level of the solid equals the redox potential of the solution. Schematically this is shown by a bending of the

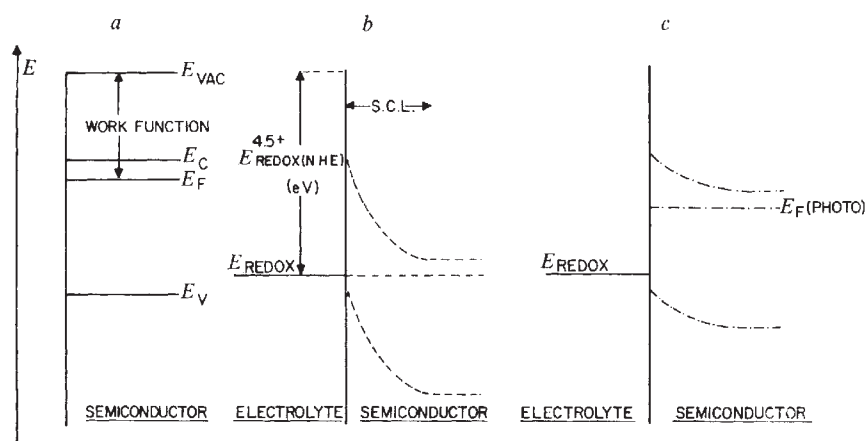


Fig. 1 Plots of energy levels: a, n-type semiconductor *in vacuo*; b, n-type semiconductor/electrolyte in the dark; c, n-type semiconductor/electrolyte when illuminated. SCL: space charge layer; E_c : bottom of conduction band; E_v : top of valence band; E_{vac} : energy level of electron in vacuum; E_F : Fermi level; E_{redox} : Redox potential of electrolyte; NHE: Normal hydrogen electrode; $E_F(\text{photo})$: Fermi level of illuminated electrode.

valence and conduction bands towards the semiconductor surface, forming the space charge layer.

Illumination by radiation with energy content more than $E_c - E_v (= E_g)$, the optical bandgap, leads to charge creation and because of the potential difference across the space charge layer, majority carriers (electrons in Fig. 1c) move towards the bulk of the semiconductor electrode and minority carriers (holes in Fig. 1c) towards the electrode surface. This movement counteracts the band bending (that is, causes a partial return to the charge carrier distribution of Fig. 1a) and creates a new situation in which the semiconductor Fermi level is no longer equal to the redox potential of the electrolyte solution. If now the semiconductor electrode is connected with a counter-electrode having the properties defined in the previous section, a photopotential V is established between the two electrodes, which can reach a maximum value of $E_F(\text{photo}) - E_{\text{redox}}$ (Fig. 2).

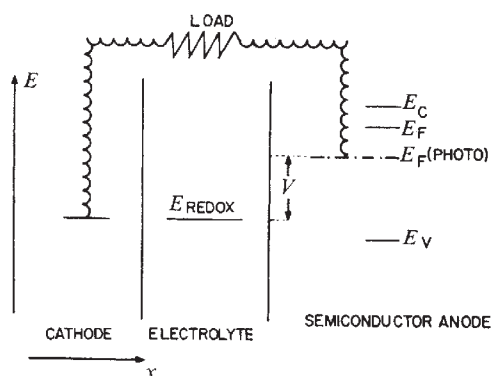


Fig. 2 Schematic illustration of the photopotential (V) obtained in a photoelectrochemical cell. The relative positions of E_c , E_F , $E_F(\text{photo})$ and E_v , from Fig. 1 are indicated.

In a working, corrosion-free cell, several kinds of energy losses occur which can be estimated, using Fig. 2 as a guide. The energy balance can be composed of the following units

$$h\nu = E_c - E_v = (E_{\text{redox}} - E_v) + (E_F - E_F[\text{photo}]) + (E_c - E_F) + iR + \eta_c + \eta_p + V \quad (1)$$

where iR are resistance losses in the system, η_c and η_p are overpotentials at the counter electrode and the photoelectrode, respectively.

We shall now make conservative numerical estimates of the different factors involved:

The difference in energy between the potential of the redox couple and the valence band, $(E_{\text{redox}} - E_v)$, has to be chosen equal to or larger than λ . Here λ is the reorganisation energy, which has been discussed extensively elsewhere¹⁴⁻¹⁶. It has to be introduced, because when $(E_{\text{redox}} - E_v) < \lambda$ the possibility exists of minority-carrier (hole, in this case)-injection from the redox couple into the semiconductor valence band, which runs counter to the effect we are looking for and may also cause corrosion of the semiconductor.

The value of λ has been estimated variously between 0.4 and 0.8 eV^{16,17} and we shall take it here at 0.4 eV.

The difference in energy between the Fermi level *in vacuo* and the Fermi level in contact with electrolyte under illumination, $(E_F - E_F[\text{photo}])$, has to have a non-zero value in order for charge separation to occur. Its allowed value will depend on the width of the space charge layer and the lifetime and mobilities of the charge carriers. To get practical currents, its value may be conservatively estimated at 0.1 eV.

The difference in energy between the conduction band and the Fermi level, $(E_c - E_F)$, may be small, but may also be several tenths of an eV (for TiO_2 it may vary between 0.003 and 0.1 eV (ref. 18)).

The terms iR and η_c can be minimised by practical cell construction and we estimate their combined values, together with that of $(E_c - E_F)$ to be 0.1 eV, which is a conservative estimate indeed.

We have shown in the preceding section that η_p cannot be too small, because of the demand of a slow back reaction and because redox reactions at semiconductor electrodes tend to be slow. Therefore η_p has to be given a larger value and we put it at 0.4 eV.

By substituting these estimates in formula (1), we get

$$h\nu = E_c - E_v = V + 1 \text{ eV} \quad (2)$$

In other words, the difference between the obtainable photopotential and the optical bandgap of the semiconductor is at least 1 eV in a working photoelectrochemical cell.

This conclusion has some important consequences. (1) If we want to use a semiconductor with an optical bandgap, so as to give optimal utilisation of solar radiation (~ 1.4 eV: ref. 17), the maximum photopotential attainable will be ~ 0.4 eV. The maximum energy obtainable from solar radiation is about 100 mW cm^{-2} . A PEC converting 10% of this energy would produce 10 mW cm^{-2} , which at 0.4 V means a current of $\sim 25 \text{ mA cm}^{-2}$. Such a current makes our estimates for the iR and overpotential losses somewhat optimistic. (2) If our aim is to decompose water, V has to be > 1.23 eV and the optical bandgap of the semiconductor electrode > 2.23 eV. Therefore to decompose water into H_2 and O_2 we have to accept the loss of an important part of the solar spectrum ($\lambda > 550 \text{ nm}$). It is possible to get around this problem by using both an n-type semiconductor photoanode and a p-type semiconductor photocathode, in which case two photopotentials, V_c and V_a (see Fig. 3) are obtained, which are additive²⁰.

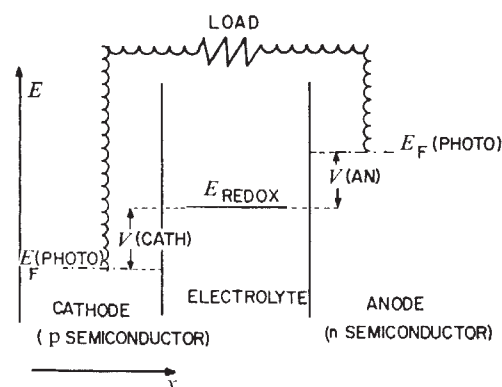


Fig. 3 A schematic illustration of the photopotential obtainable when a combination of a photoactive n-type anode and a photoactive p-type cathode is used.

It is interesting that the water decomposition reaction, which occurs naturally in green plant and algal photosynthesis using chlorophyll, also requires two light quanta to obtain the necessary electrochemical potential difference, and that of the light input of 1.8 eV of each quantum, only 1 eV is utilised for the generation of an electrochemical potential difference²¹.

Most of the photoelectrochemical cell systems described are complicated by corrosion problems and its prevention is one of the most difficult obstacles to practical realisation of this system. The Cd-chalcogenide based cell, in which the corrosion problem is circumvented by the use of the S/S^{2-} redox couple, shows great promise in this respect^{22,23}.

Corrosion

It is clear that the electrodes and the redox couple must be entirely stable in the environment of the electrolyte used. There

is however an additional phenomenon, that of corrosion under the influence of light. With CdS the S^{2-} ions are oxidised to sulphur and with ZnO the O^{2-} ions are oxidised to oxygen and the Zn^{2+} ions pass into the solution^{24,25}. This means that both photoactive anodes are not stable towards the strong oxidising conditions which exist at their surfaces during illumination. On the other hand, TiO_2 has been shown to be stable under these conditions, as has WO_3 (ref. 26) and Ta_2O_5 (ref. 27). Both cadmium and zinc have only one stable valence state, whereas titanium, tungsten and tantalum are stable in several valence states. It may be, therefore, that one way to obtain stability against photocorrosion in these conditions is by the presence of ions in the semiconductor which can accommodate several valence states.

Photochemical aspects

Problems connected with the heterogeneity of solar radiation have been extensively discussed elsewhere and need not be dealt with here¹⁹.

One has to realise however, that for reasonable conversion efficiency, the light has to be absorbed within the space charge layer, where the necessary potential gradient exists for charge separation to occur. For a material having a decadic absorption coefficient α , 90% of the incident radiation will be absorbed in a layer of thickness

$$L = 1/\alpha \quad (\alpha \text{ in cm}^{-1})$$

A value for α of 10^4 – 10^5 cm^{-1} is typical of many semiconductors in the strongly absorbing range (both TiO_2 and Ta_2O_5 have exceptionally high absorption coefficients close to 10^6 cm^{-1}) and this would demand a thickness of the space charge layer of about 10^3 – 10^4 Å. To minimise ohmic losses, we should like to make the semiconductor electrode as conductive as possible. As the space charge layer narrows with increasing conductivity, it can easily reach a stage, at which most of the light passes the space charge layer, with a resultant decrease in conversion efficiency.

Technological and economic aspects

The terms photoelectric energy storage and photoelectric energy conversion are sometimes confused.

A photoelectric energy converter produces electrical energy under illumination and does not store it unless specific storage electrodes can be used²². As long as energy production by such a device is small, compared with the overall use of electricity, the electrical current produced can be fed directly into the existing distribution system. This will save fuel, but the capacity of alternative generating capacity will have to remain the same to prevent serious shortages on cloudy days. With the increasing emphasis on the use of nuclear energy, fuel saving will become less important, since fuel costs in a nuclear power station are much smaller than those in a conventional station. Better and cheaper means of storing electricity, however, remain desirable, and hence the practical importance of photoelectrochemical decomposition of water into hydrogen and oxygen.

We have shown that to achieve this with the use of only the photoactive electrode is very inefficient, (no examples have as yet been given in the literature to show that this is feasible without adding energy to the system^{7,10,11}). It must be kept in mind that if hydrogen can be produced by the use of a photoactive anode and a reversible counter electrode, most of the energy from hydrogen production can be converted into electrical energy by the use of an oxygen cathode. Fuel cell technology has produced oxygen cathodes with low overpotentials for oxygen reduction under the conditions of low current density which invariably will exist in a solar energy device which does not concentrate the sunlight.

Electrical current may be transported to a central electrolysis

plant, which converts water into hydrogen and oxygen at a high efficiency (90% has been claimed²⁸). This has to be carefully weighed against the intrinsic lower efficiency of hydrogen production and the appreciable technological difficulties in transporting small quantities of hydrogen (70 ml of hydrogen per (mA cm^{-2}) per min per m^2 of electrode area) leakfree to a central container. If a combination of an n-type and p-type semiconductor is used, both have to be exposed to sunlight. This is only attractive when both photoactive electrodes work at about the same efficiency. If one of the two is appreciably more efficient, it is again more favourable to use the most efficient electrode for electricity production alone and to produce the hydrogen in a central electrolysis plant. The only way out of this dilemma would be to use one electrode which absorbs one part of the solar spectrum and positioning it in front of another electrode, which responds to the radiation which has passed the first one.

It seems therefore that the technological prospects of photoelectrochemical decomposition of water are rather dim because there is always the alternative of producing electrical current instead at a higher efficiency and using the current for electrolysis of water in a central plant.

Advantages of photoelectrochemical cells

In the above discussion several inherent disadvantages of the photoelectrochemical cell have been emphasised, which may be less severe for dry photovoltaic devices. There are several arguments however, in favour of pursuing research and development of the photoelectrochemical cell. It has been shown that high efficiency can be obtained, using a thin polycrystalline semiconductor surface. This is because of the much better contact obtained between a polycrystalline solid and a liquid than is possible between two polycrystalline solids, and the "single crystal barrier", which is often used as an argument against cheap solid-state solar cells, does not have to be overcome here. Therefore the production cost of a photoelectrochemical cell can be considerably less than that of dry cells. "Cost" does not only mean the money to be paid, but also the energy-input in production. By mass-production techniques it will certainly be possible to decrease the cost in terms of money of dry photovoltaic cells, but it is much more difficult to bring down the cost in terms of energy and it is in this respect that the photoelectrochemical cell has a chance to compete with dry photovoltaic devices.

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articles

γ -ray bursts from thermonuclear explosions on neutron stars

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It is proposed that γ -ray bursts originate from carbon detonations initiated by the accretion of matter on to the surface of a neutron star. The observations are interpreted in terms of this theory. Possible implications for the nuclear powered model of giant X-ray pulses are discussed briefly.

MORE than three years have now elapsed since the first published report of cosmic γ -ray bursts by Klebesadel *et al.*¹, but in spite of continuous monitoring and observation^{2,3} the astrophysical origin of these phenomena still remains enigmatic⁴. Briefly, the dominant characteristics of the observed bursts are:

(1) Total durations of ~ 1 – 10 s with significant time structure on scales as short as 16 ms.

(2) Integrated burst energies at the earth of 10^{-5} – 10^{-4} erg cm^{-2} for events which occur ~ 10 times per yr, with some evidence for more frequent less energetic bursts of $\sim 10^{-7}$ erg cm^{-2} occurring perhaps several times per d (refs 3, 5).

(3) An energy spectrum that can be represented by an exponential from 100 to 400 keV tangent to a power law at higher energies³. The spectrum seems typical of a cooling black body of a temperature $\sim 2 \times 10^9$ K (ref 6).

(4) The distribution of energetic sources seems to be crudely isotropic and uncorrelated with the observed strong X-ray sources, although Cyg X-1 is a possible exception².

The short time scale structure of these bursts suggests that they originate from a compact object⁷, and the spectrum indicates a possible thermal source. We propose that the observed bursts are produced on the surfaces of neutron stars as a result of violent thermonuclear runaways initiated in accreted nuclear fuels. Attention is focused on the onset of carbon burning which we find, for reasonable choices of parameters, occurs in conditions that are very degenerate and potentially quite explosive. These same conditions have previously been found to result in the detonation of carbon in the core of highly evolved stars and have been investigated as a possible mechanism for the supernova phenomenon^{8–13}. Such detonations are characterised by very short time scales and multibillion degree temperatures. Here, by using the gravitational potential well of a neutron star, we find that similar behaviour can occur for critical carbon masses of only 10^{23} g as opposed to the $1.4M_{\odot}$ usually required for a detonation initiated by self-gravitation.

The model

Consider a $1.4M_{\odot}$ neutron star of radius 10 km accreting hydrogen and helium at $10^{-10}M_{\odot} \text{ yr}^{-1}$. An accretion rate of this magnitude might be realised by a Roche lobe overflow of a less massive main sequence companion or perhaps by a single neutron star in a very dense cloud or nebula. This object would not appear as a strong X-ray source, and, because of

the softness of the resulting spectrum¹⁴, might be difficult to detect at a distance of, say, 1 kpc. It will turn out that accretion rates of roughly this magnitude are required since the proposed detonation does not seem likely for markedly different values.

If the host neutron star has a strong magnetic field, the material is not accreted in a spherically symmetric manner, but instead accumulates in polar caps of $\sim 10^{10} \text{ cm}^2$ or $\sim 0.1\%$ of the surface area^{4,15}. Initially any nuclear reactions that occur in this matter act only to strip down nuclei and fragment them. As additional material is accreted, however, compression occurs and nuclear fusion reactions, enhanced by electron screening, commence. Hydrogen burns to helium^{15,16} and helium to carbon and oxygen¹⁶. These burning phases may become unstable, but, whatever the outcome, it is clear that little material escapes the gravitational field of the neutron star. Hansen and Van Horn¹⁶ have found that the density of the helium-burning shell on a neutron star is $\sim 10^6 \text{ g cm}^{-3}$. Thus one expects that the carbon formed by this burning will be relativistically degenerate. Because of the high conductivity of degenerate matter, the carbon will be maintained at a temperature close to that of the helium-burning shell. For the assumed accretion rate and geometry one can use the Hansen and Van Horn calculations of nuclear fusion on neutron stars to obtain an estimate for this temperature of $\sim 4 \times 10^8$ K (Note that, in terms of accretion per unit area, $\dot{M} = 10^{-10} M_{\odot} \text{ yr}^{-1}$ on the poles is equivalent to a uniform global rate of $\dot{M} \approx 5 \times 10^{-8} M_{\odot} \text{ yr}^{-1}$.)

It is of some interest to examine the state of this carbon layer after 4×10^{23} g of matter has accumulated: the density at the base of the carbon shell will then be $\rho_b \sim 2 \times 10^9 \text{ g cm}^{-3}$ (calculated from the equation of hydrostatic equilibrium with pressure support from degenerate electrons). This shell of carbon, ~ 50 m thick, is capped by 1 or 2-m layers of hydrogen and helium containing $\sim 10^{16}$ and $\sim 10^{17}$ g respectively, both with active burning shells. Before reaching this density, the carbon layer is cooled by neutrino emission, with maximal losses in the regions of highest density. A positive temperature gradient will develop, so that conduction acts to heat the dense carbon rather than cool it. Once a density of $\sim 10^9 \text{ g cm}^{-3}$ is reached, however, the plasma neutrino losses become rather suddenly less effective in cooling, and at a density $\sim 2 \times 10^9 \text{ g cm}^{-3}$ the nuclear energy generation from carbon burning dominates the neutrino losses. Any increase in density above this value leads to a nuclear runaway^{8,9,17}. Because of the high degree of degeneracy, low specific heat, and extreme temperature sensitivity of the carbon-burning reaction the runaway will be quite violent. In the carbon-detonation supernova model, carbon ignites at temperatures and densities almost identical to those described here, and one achieves temperatures $> 5 \times 10^9$ K and nuclear burning time scales (once a significant amount of carbon has burned) of ~ 1 ms (ref. 8). Not only does carbon burn, but also oxygen and their products as well. Here one

expects the entire layer of carbon to burn on a very short time scale either through a series of carbon flashes¹³ or by the formation and propagation of a detonation wave¹⁰. If a detonation wave propagates the relevant time scale is < 1 ms. Ultimately, species in the iron group are produced and 7.6×10^{17} erg g⁻¹ of nuclear energy is liberated. Thus in the case of the model neutron star discussed here, complete combustion of all available carbon releases 3×10^{40} erg. This energy is dissipated through neutrino processes, conduction, convection, and the expansion of the neutron star atmosphere. But unlike the case of a supernova, which it otherwise so closely resembles, the expansion that results from an explosion on a neutron star is negligible. The total energy generated by the explosion is only $\sim 0.5\%$ of the gravitational binding energy of the carbon. Moreover, any energy that goes into expansion is immediately regained on a time scale $\sim 10^{-3}$ s as the material is pulled back by gravity. Thus, the only immediate result of a carbon detonation in this case is to heat material to a very high temperature.

The relevant time scales at this point become very difficult to estimate especially without a detailed numerical model. The incandescent carbon will cool by emitting neutrinos, by conduction to the core and surface, and by convection. The conductivity of the hot carbon ($T \sim 5 \times 10^9$ K) is not as high as it was before the explosion (since $K_{\text{cond}} \propto T^2$). We estimate the thermal relaxation time scale for the 50 m of carbon to be $\sim 10^3$ – 10^4 s if cooling occurs by conduction alone, but this is an overestimate since convection will undoubtedly have an important role. Even with a detailed numerical model the physics of convection in a relativistically degenerate electron gas permeated by a 10^{12} -gauss magnetic field would be very uncertain. All one can say at this point is that a cooling time scale of a few seconds does not seem unreasonable.

The emission from the stellar surface may also have an unusual character. As mentioned previously, the layers of hydrogen and helium capping the carbon are very thin, and contain only $\sim 10^{-6}$ of the mass of the exploding carbon. It appears very likely that instabilities and convective overshoot will develop so that hot blobs of burning carbon will break through the surface of the neutron star. There incandescent blobs having temperatures of several billion degrees and dimensions perhaps of several metres will cool by emitting neutrinos and γ rays in times < 1 s. Additional temporal structure may result if the neutron star is an oblique rotator with rotation period less than the cooling time.

Comparison with observations

How do these results compare with the observations? The spectrum, following the above discussion, is that of a rapidly cooling multibillion degree black body, and the short time scale structure in the observed bursts relates to the cooling times of these small hot convective blobs. If one assumes that only 10% of the available energy is radiated in this form, the total photon energy is $\sim 5 \times 10^{39}$ erg. At a distance of 1 kpc this would give rise to an integrated flux at the Earth of $\sim 3 \times 10^{-5}$ erg cm⁻² in good agreement with observations.

How many such objects would exist within 1 kpc of the Earth? Various arguments involving nucleosynthesis, supernova rates, and pulsar statistics suggest that there are $\sim 3 \times 10^8$ neutron stars in the entire Galaxy. If these are distributed uniformly throughout the Galaxy with a scale height $\lesssim 1$ kpc above the galactic plane one can expect $\sim 10^6$ neutron stars within 1 kpc of the Earth. Of these, perhaps 1% are in binaries. What fraction of the binaries are currently accreting mass at the required rate? This is connected with the mode of mass transfer which could be either a stellar wind, or Roche lobe overflow. If the former is the case, the mass transfer rate from the companion must be $\gtrsim 100$ times the accretion rate, since the neutron star subtends a small solid angle. This suggests that the mass loss rate from the companion is $\sim 10^{-8} M_{\odot}$ yr⁻¹ which is typical for supergiant stars ($M \gtrsim 20 M_{\odot}$). However the duration of this mass transfer phase is $< 10^5$ yr (ref. 18). One then expects $10^6 \times 0.01 \times 10^{-5} < 1$ object at this phase

of evolution. Clearly this is not in accord with the observations. If one considers Roche lobe overflow, a mass loss rate of $10^{-10} M_{\odot}$ yr⁻¹ might be typical for a main sequence star less massive than the neutron star transferring mass on a nuclear time scale. One would then expect $10^6 \times 0.01 \times (0.01-0.1) \sim (100-1,000)$ objects. If the neutron star was formed by a type II supernova, there would be some doubt as to whether the 'desired' system would remain bound. For progenitor masses $\gtrsim 4 M_{\odot}$ (believed to be a probable lower limit) the binary would probably disrupt since the amount of mass loss in the supernova explosion would be $> \text{half}$ the total mass of the initial system¹⁹. These arguments preclude the existence of γ -ray burst sources as evolving from type II supernovae. It is, however, not implausible that the systems could have evolved from type I supernovae (as, for example, may occur in cataclysmic variable systems)²⁰. Accepting the above conclusion, one might expect the sources to be confined to the galactic plane, since the progenitors of the type I supernovae are believed to be old stars in the stellar disk. After the supernova explosion, however, there would probably be a random distribution of systems reflecting the randomness in the initial orientation of the binary orbits. Since these neutron stars could have been given high peculiar velocities and were produced as long as a billion years ago, a scale height of 1 kpc is not unreasonable. Thus we conclude, admittedly with a great deal of uncertainty, that 100 such objects could currently be active within 1 kpc of the Earth. Since a critical mass of carbon will accrete every year (giving rise to the possibility of repeated bursts from the same location) the number of events with an integrated flux in the range 10^{-5} – 10^{-4} erg cm⁻² would be ~ 100 yr⁻¹. Even allowing for many events that go undetected this satisfies the observed frequency requirements. The large scale heights of these objects above the galactic plane would also result in a roughly isotropic distribution for the strong events. At lower energies one might begin to see some association with the galactic disk depending on the actual scale heights involved. We would predict roughly 100 events per d having total integrated flux $> 10^{-7}$ erg cm⁻².

What is the fate of neutron stars that accrete at rates differing markedly from $10^{-10} M_{\odot}$ yr⁻¹? While there may be interesting activity associated with semi-degenerate shell flashes in such stars, we do not envision ¹²C detonation as occurring. For a very low accretion rate, $\dot{M} < 10^{-11} M_{\odot}$ yr⁻¹, the energy generation rate from carbon burning is so slow that a runaway cannot occur even when nuclear energy generation exceeds neutrino losses. Electron conductivity is very efficient in such cases and using the criterion of Giannone and Weigert¹⁷ it seems that no runaway occurs. Instead, the carbon and oxygen are neutronised at $\rho \sim 2 \times 10^{10}$ g cm⁻³. We note, however, that a very uncertain electron screening factor has a dominant role in this statement. For much larger accretion rates, $\dot{M} > 10^{-9} M_{\odot}$ yr⁻¹ (for the assumed geometry), the temperature of the helium-burning shell approaches 10^8 K (ref. 16) so that carbon can ignite before becoming extremely degenerate and a detonation will not ensue. In this regard, the accretion rate plays a similar role to that of the mass of the star in ordinary stellar evolution. It is well known that a massive star, $M \gtrsim 10 M_{\odot}$, will ignite carbon in its core non-degenerately, whereas in a less massive star, carbon will ignite explosively in highly degenerate conditions²¹.

Giant X-ray pulses?

We note finally the possible relationship of the above model for γ -ray burst production to the recently observed giant X-ray pulses (see discussions in ref. 22). Although the observations are difficult to interpret at this early stage we feel that two categories of X-ray bursts are being observed: aperiodic (without periodicity) and quasi-periodic. We believe, in agreement with W. D. Evans, R. D. Brian and J. P. Conner (unpublished), that the aperiodic bursts are the soft energy tails of the spectra of distant γ -ray burst sources. In the preceding section it was suggested that a plausible number of such distant

outbursts for our Galaxy was $\sim 100 \text{ d}^{-1}$. The distribution of these bursts should correlate with the galactic plane and might have a tendency to peak towards the galactic centre where the spatial density of neutron stars is expected to be higher.

The quasi-periodic sources pose a greater problem. Periodicities as short as $\sim 15 \text{ s}$ have been observed which rule out any model based on carbon detonation. In fact, unless the neutron star can somehow act as a storage battery, a simple calculation shows that bursts with periods of $\lesssim 30 \text{ s}$ and durations of 1 s cannot be seen above the steady-state accretion luminosity for any nuclear powered model. This follows from the fact that gravitational accretion liberates 10–20% of the rest mass energy of the accreted material while all subsequent nuclear reactions generate $< 0.7\%$. Thus even if hydrogen explodes and produces X rays with 100% efficiency the 15-s observations are very difficult to interpret in terms of a flash that burns only the material accreted during the interflash period. We believe, however, that these rapid pulses can result from a series of helium (and perhaps carbon) shell flashes occurring in semi-degenerate material with a total mass $\sim 10^{22} \text{ g}$ that has accreted over a period of time that is long compared with the interflash period. (Admittedly, it might be difficult to accumulate 10^{22} g without the nuclear burning having already occurred.) The neutron star stores the potential nuclear energy for a time during which its entire output is from the release of gravitational energy and then liberates it in a series of rapid flashes. More violent flashes may heat up the material to such an extent that the diffusion of energy outwards (and hence the return to an unstable configuration) takes longer than for weaker flashes. The fact that the time between bursts is longer for the more energetic bursts is an observed attribute of certain X-ray burst sources²³. Clearly a large range and diversity of nuclear outbursts

of various energies and time scales are possible for the possible spread of accretion rates, neutron star masses, and magnetic field configurations. The subject is ripe for continuing experimental investigations and serious theoretical examination, and hopefully this somewhat speculative paper will encourage both. We especially urge that known γ -ray burst sites be continuously monitored for giant X-ray pulses and vice versa.

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Tritium inventories of the world oceans and their implications

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Tritium inventories are given for the Pacific Ocean from 51°N to 60°S. As expected, the highest inventories are found north of 20°N. Inventories drop rapidly from 20°N to 10°N. From 10°N southwards, inventories in the top 500 m are essentially constant. From these data, it is calculated that $107 \pm 21 \text{ kg}$ of tritium were present in the Pacific Ocean in 1970. A total of $\sim 300 \pm 80 \text{ kg}$ of tritium was present on the Earth's surface in 1970. This indicates that $550 \pm 160 \text{ kg}$ of tritium have been produced by nuclear detonations. It is estimated that 62% of the tritium in the Pacific Ocean was added through molecular exchange, 5% through runoff from the continents, and 33% as precipitation.

TRITIUM ($t_1 = 12.3 \text{ yr}$) is present on the Earth's surface mainly as HTO. The only significant natural sources are cosmic rays passing through the Earth's atmosphere and possibly accretion from the solar wind. An equilibrium quantity of $\sim 3.5 \text{ kg}$ is present from these sources¹. Another source of tritium is the nuclear reactor. This source has some effect on the content of atmospheric hydrogen, but accounts for only minor amounts of HTO^{2,3}. The major source of the tritium now present on the Earth has been the detonation of fusion bombs. The amount

of tritium produced by nuclear explosions is about two orders of magnitude higher than that produced by all the other sources. Estimates of the amount have varied from 800 kg (ref. 4) to 200 kg (ref. 5).

Most tritium has been deposited in the world's oceans by one of three processes: precipitation as rain or snow, runoff from the continents, or molecular exchange. Precipitation has been monitored in detail by the International Atomic Energy Agency and continental runoff is small in comparison. The amount of tritium added through molecular exchange is not well known^{5–10}.

The La Jolla Tritium Laboratory has studied and used tritium as an oceanographic and geochemical tracer since the late 1950s. From 1968 to 1972, several series of depth profiles from various locations in the Pacific Ocean were analysed for tritium (Fig. 1). The oceanographic implications have been discussed elsewhere¹¹, but here we use the observations to study geochemical problems such as molecular exchange and tritium inventories.

Concentrations in top 500 m of ocean

Table 1 lists inventories of tritium expressed as numbers of atoms km^{-2} in the top 500 m at various profile stations. The 500-m surface layer is used. Tritium below that depth, if present in all measurable quantities, is brought in by lateral flow

Table 1 Tritium profile inventory in the top 500 m of the Pacific

Cruise	Year	Position	Inventory (10^{19} atoms km^{-2})
Seven Tow	1970	50°52'N 171°31'W	23.4
Seven Tow	1970	44°59'N 167°08'W	32.0
OSN	1972-73	30°N 140°W	29.7
Seven Tow	1970	28°01'N 156°01'W	21.0
La Pared	1965	27°44'N 118°03'W	14.5
Seven Tow	1970	22°49'N 157°12'W	29.4
La Pared	1965	21°00'N 119°14'W	13.8
La Pared	1965	19°28'N 116°02'W	10.3
Seven Tow	1970	18°09'N 169°01'W	25.4
Scan	1969	15°22'N 169°33'W	23.1
Seven Tow	1970	13°47'N 166°40'W	15.6
South Tow	1972	10°18'N 123°10'W	2.4
Scan	1969	9°58'N 151°31'E	6.3
Scan	1969	9°30'N 166°31'E	4.6
Scan	1969	9°20'N 179°56'E	5.6
Seven Tow	1970	8°55'N 158°35'W	5.6
South Tow	1972	5°13'N 124°32'W	5.0
Seven Tow	1970	3°13'N 164°48'W	6.6
South Tow	1972	0°28'N 125°31'W	4.6
South Tow	1972	4°59'S 126°25'W	2.7
South Tow	1972	9°59'S 127°22'W	3.1
South Tow	1972	29°27'S 144°40'W	4.4
South Tow	1972	39°45'S 137°58'W	3.2
South Tow	1972	40°39'S 93°06'W	2.6
South Tow	1972	50°57'S 118°47'W	2.5
Eltanin	1972	54°07'S 172°55'E	3.9
Eltanin	1972	59°37'S 171°13'E	2.8

from polar latitudes and is not a result of surface input at the station.

Some profiles do not go as far as 500 m down. In some of these cases, reasonable estimates can be obtained if tritium concentrations decrease rapidly with depth. In no case should the error from these estimates exceed 10%. In some cases no estimates were possible and inventories are not given for those stations (see ref. 12 for the complete listing of data).

Correlation of data with latitude

Figure 2 shows a plot of tritium inventory against latitude. Tritium inventories are relatively constant from the subarctic Pacific to $\sim 20^\circ\text{N}$, but further south they start to show a marked decrease. A minimum is reached at $\sim 10^\circ\text{N}$, and throughout the Southern Hemisphere they are almost constant. Inventories are highest at northern latitudes because of the spring leak phenomenon¹³, which results in a large input of tritium into the oceans from 30°N to 60°N . Oceanographic factors also affect the tritium distribution; tritium isolines and density isolines show strong correlations¹¹. Density isolines start rising sharply south of 20°N and rise closest to the surface at 10°N . Tritium isolines show the same trend and a minimum in tritium inventories is found around 10°N .

Inventories for tritium below 500 m are significant only in the Southern Hemisphere. For the latitudes of 30°S to 50°S , inventories for below 500 m are approximately as large as for above 500 m (refs 12, 14). In the north, the tritium present below 500 m is negligible as a fraction of the total.

As these data cover most of the Pacific Ocean from North to South, it is possible to estimate the tritium burden of that ocean. All inventories are age corrected to 1970. The inventory is calculated assuming that the tritium inventory is constant to $\pm 20\%$ over a given latitude band. Only in the extreme east at 10°N does a large deviation occur. Carbon-14 values are also lower in the equatorial eastern Pacific¹⁵ as a result of upwelling and influx of water from the South Pacific. There are two tritium profiles available from a GEOSECS station at 28.5°N (refs 16 and 17). These profiles have an average tritium inventory similar to profiles at equivalent latitudes presented here.

Michel and Suess have noted that surface tritium distributions have an east-west asymmetry with highest values in the east¹¹. Also, caesium-137 determinations have shown that this

radioisotope is mixed deeper in the west and this dilutes its surface concentration^{18,19}, and, although vertical distributions vary, inventories seem not to be significantly different. The assumption of a constant inventory on an E-W line can be assumed to be correct within $\sim \pm 20\%$.

At 28°N , one Seven Tow profile had a low inventory compared to other profiles near it. A profile taken near the same position 4 months later showed a completely different distribution, so the profile at 28°N has been deleted from calculations.

Total inventories

In Table 2, the inventories of tritium at various ranges of latitudes are given for the Pacific Ocean. The inventories include tritium below 500 m as well as in the top 500 m. It is assumed the tritium content of the deep water is negligible^{16,17}. A total of 107 ± 21 kg was found to be present in the Pacific in 1970, with $> 80\%$ of the tritium present in the North Pacific.

Table 2 Pacific Ocean tritium inventory (1970)

Latitude	Number of T atoms (10^{19} km^{-2})	$\text{km}^2 \times 10^6$	Quantity of T (g)
$< 30^\circ\text{N}$	32	30	48,000
$30^\circ\text{N}-20^\circ\text{N}$	30	14.6	21,000
$20^\circ\text{N}-10^\circ\text{N}$	15	16.6	12,500
$10^\circ\text{N}-0^\circ$	5	20.1	5,000
$0^\circ-30^\circ\text{S}$	4	49.8	10,000
$30^\circ\text{S}-50^\circ\text{S}$	5.8	26.6	7,700
$< 50^\circ\text{S}$	3	19	1,900
			Total $107,000 \pm 21,000$ g

Some idea of the total tritium present on the surface of the Earth can be obtained from these data and data of other researchers on tritium in other large bodies of water^{14,20-23}. Most other work has been on the North Atlantic. The Atlantic north of 30°N has a consistently higher tritium inventory per square kilometre than the Pacific. South of 30°N inventories are similar. In calculating total inventories, the Atlantic is found to have an inventory 1.6 times as large as the Pacific, north of 30°N , and equal to the Pacific south of 30°N . Measurements are very sparse in the South Atlantic and inventories are assumed to be similar to those in the South Pacific at similar latitudes.

No tritium data are available from the Indian Ocean. It is assumed that inventories in the Indian Ocean are similar to those of the Pacific at similar latitudes. Most of the Indian Ocean surface area is south of the Equator where the tritium concentrations are low. Thus, the error in estimating the Indian Ocean inventory is small in terms of the total inventory. The Arctic Ocean inventory is calculated entirely on the basis of input by precipitation derived from the data of Schell and Sauzay²⁴. Any input into the Arctic by molecular exchange should be small, as ice cover inhibits this process (R. Weiss, personal communication). Therefore it seems that the total tritium in the world's oceans is 250 ± 50 kg (Table 3).

Continental tritium

A small fraction of the Earth's water supply is found in the active freshwater bodies of the continental land masses.

Table 3 World inventory of tritium (1970)

Pacific Ocean	107
Atlantic Ocean	120
Indian Ocean	20
Arctic Ocean	6
Worlds Oceans' Total	250 ± 50 kg
Land water	45 ± 25 kg
Air (HTO and HT)	3 ± 11 kg
Total	300 ± 80 kg

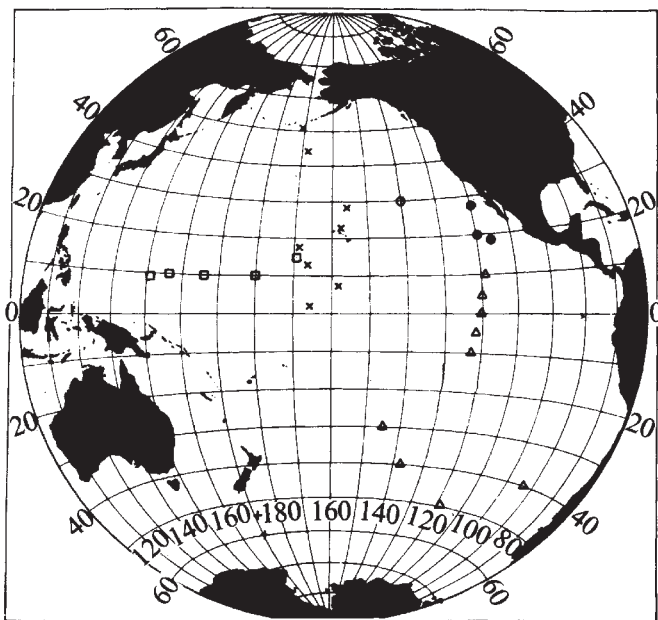


Fig. 1 Positions where tritium depth profiles were collected by the La Jolla Tritium Laboratory. ●, La Pared (1965); □, Scan (1969); ×, Seven Tow (1970); △, South Tow (1972); +, Eltanin (1972); O—OSN (1972).

Because of the continental effect on the tritium in rain, however, tritium concentrations are much higher in these water masses. Estimating the tritium inventory of the continental water masses is difficult, as concentrations there are much less uniform than in oceanic water masses. Also, very few data are available for 1970. It is possible to obtain an estimate of the tritium on the continents in the mid-1960s (ref. 25) which can then be age-corrected to 1970. Runoff and precipitation will cause some fluctuation in the inventory, but these effects will be small. It appears that there were 45 ± 25 kg of tritium on the continents in 1970. The tritium content of air is known more accurately^{2,3}, and can be estimated to be 3 ± 1 kg most of which is in the form HT.

Total tritium

These inventories give a total of 300 ± 80 kg of tritium present on the surface of the Earth in 1970, most of it produced by nuclear testing.

From the world inventory, the total tritium produced by the early 1970s by nuclear explosions is calculated to be 550 ± 160 kg,

Table 4 Modes of tritium input into the Pacific ocean (1970)

Precipitation	36 kg
Evaporation	−3 kg
Runoff	6 kg
Molecular Exchange (calculated)	68 kg
Total (from Table 2)	107 kg

where it is assumed that tritium production has been proportional to the megaton yield of nuclear explosives. Knowing the history of nuclear weapons testing, it is possible to correct for tritium decay⁵.

This estimate may be low as it is assumed that no tritium is present in the deep water of the Pacific and Indian Oceans. Even a small concentration of tritium in the deep water would, however, result in a significant increase in the inventory. If the experimental upper limit of 0.2 TU (1 TU is one tritium atom per 10^{18} hydrogen atoms) is used as the deep-water concentration, the figure for tritium produced by nuclear weapons testing would be 130 kg higher.

Molecular exchange

As mentioned previously, the extent of the contribution of molecular exchange to the input of tritium into the oceans is still uncertain. It was postulated^{5,6} that most tritium in the oceans is added by molecular exchange. In a more formal treatment, Craig and Gordon⁷ came to essentially the same conclusion. This seemed, however, not to be in agreement with experimental data. Two ocean studies^{8,9} found experimentally that molecular exchange added < 0.33 of the tritium present. But the measurements were carried out in areas of low humidity where molecular exchange is expected to be low. Simpson¹⁰ also found that molecular exchange could account for only about one third of the tritium added to Crater Lake. Conditions for this lake, however, are substantially different from those prevailing over the oceans, and the results are not necessarily significant for this problem.

Our data can be used to calculate the large scale molecular exchange for the Pacific Ocean. The only functions which affect tritium inventory are precipitation (P), evaporation (E), decay, runoff (R) and molecular exchange (ME). With the data corrected for decay, input by molecular exchange can be found by

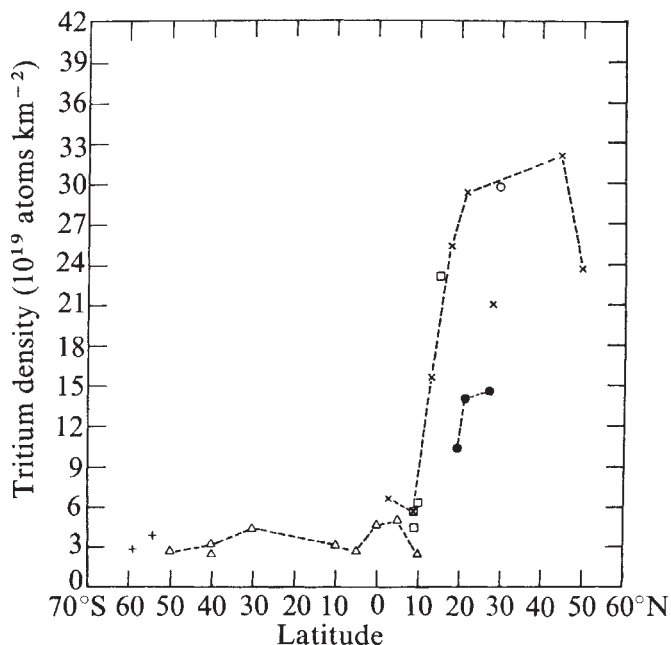
$$ME = I - P - R + E \quad (1)$$

where I is the inventory in 1970 (107 kg).

The amount of tritium deposited in the Pacific since 1963 can be calculated from the data of Schell and Sauzay²⁴. Rainfall for most of the North Pacific is taken from data compiled by Reed and Elliott²⁶. Rainfall data south of 20°N and evaporation data are taken from Sverdrup *et al.*²⁷. Tritium concentrations at the sea surface are well known in the Pacific^{12,28} so evaporation loss can be evaluated. The relative amounts of tritium added before 1967 have been calculated by Roether²². These data combined with the input data of Schell and Sauzay²⁴ have been used to estimate the input before 1963. All input by precipitation was corrected to establish the tritium deposited by 1970, and the net input was found to be 33 kg.

Approximately 9×10^{15} l of water enter the Pacific Ocean each year as runoff. Estimates of the tritium content of runoff are available from International Atomic Energy Agency (IAEA) data²⁵. In calculating total runoff, it is assumed that runoff is of equal volume in all latitude bands from 50°N to 50°S . In

Fig. 2 Inventory of tritium in the top 500 m of the Pacific Ocean km^{-2} . Symbols as for Fig. 1.



latitudes where no tritium data are available, it is expected that variations in tritium concentration in runoff are similar to the variations in rainfall. The errors are large but runoff tritium is small compared to input by molecular exchange or precipitation, being responsible for ~ 6 kg of the 1970 inventory. The input by molecular exchange is then found to be 68 kg (Table 4). This is 62% of the total tritium input in the Pacific—in agreement with previous estimates—and almost twice the input by precipitation alone. Thus, molecular exchange is indeed the predominant mechanism by which tritium enters the oceans.

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Rolling hairpin model for replication of parvovirus and linear chromosomal DNA

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A novel, quasicircular scheme is proposed for the replication of parvovirus DNA. Daughter strands are initiated after the copying and rearrangement of a terminal palindromic sequence, a process termed 'hairpin transfer'. Such a process may be involved in the replication of other viruses and host cell DNA.

ALL known DNA polymerases require a 3'-OH terminus on which to initiate strand elongation^{1–3}. Although a satisfactory mechanism for the complete replication of circular DNA molecules can be envisaged by using RNA primers³, additional molecular conjuring is required to explain the completion of the 5' termini of daughter strands in a linear DNA molecule. The problem arises because excision of a terminal RNA primer leaves a 5' terminal gap and because no polymerase is known which catalyses DNA chain growth in a 3'→5' direction. Several elegant models have been put forward to circumvent the difficulty, all requiring special terminal structures, for example, terminal redundancy⁴, palindromic termini⁵ and covalently continuous (cross-linked) termini⁶. It has not been feasible to determine whether such terminal structures exist in the DNA molecules of eukaryotic cells, because of their extremely high molecular weight⁷. But practicable model systems for the study of chromosome structure and replication are provided by animal viruses with linear DNA genomes. For example, the parvoviruses contain linear single-stranded DNA genomes of only 1.4×10^6 – 1.7×10^6 daltons^{8,9}, which replicate

by way of linear double-stranded intermediates^{10–18}. They are divided into two subgroups. The members of one subgroup, the adeno-associated viruses (AAV), are entirely dependent on adenovirus coinfection for their own replication, and package both plus and minus DNA strands in separate virions^{8,9}. Members of the other subgroup, the autonomous parvoviruses, can replicate without a helper virus and package a unique viral DNA strand⁸.

Structure of the parvovirus genome

To determine whether special arrangements of nucleotide sequence are necessary for the replication of a linear DNA molecule, we have examined the structure of the genome of the minute virus of mice¹⁹ (MVM), an autonomous parvovirus. The results of this study²⁰ are summarised in Fig. 1. We have shown²⁰ that the 5' end of the molecule is a hairpin, represented diagrammatically by the sequence ABA. The duplex Aa can be isolated as an S1 nuclease-resistant, hydrogen-bonded fragment about 130 base pairs long, containing the 5' terminal base. The sequence represented by D is about 95% of the total genome. It is entirely single stranded and approximately 4,100 bases long. The 3' end can also form a hairpin denoted EFe, which serves as a primer for the synthesis of the complementary strand *in vitro* by a number of DNA polymerases. Various oncornavirus DNA polymerases (reverse transcriptases) can use MVM DNA efficiently as a primer-template. Since these polymerases lack exonuclease activity^{21,22} and require base-paired primers^{21–23}, this implies that the 3' terminal base is hydrogen bonded in the duplex Ee. The length of this duplex is not precisely known, but it does not seem to be stable at

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37 °C *in vitro*, as the entire genome, excluding the 5' hairpin, is sensitive to *E. coli* exo I (ref. 20). In addition, its priming ability is not destroyed after treatment with *E. coli* exo III.

Polymerases lacking 5' to 3' exonuclease activity synthesise a duplex DNA with a molecular weight 1.96 times that of the viral genome, in which the complementary strand is covalently attached to the template²⁰. Restriction enzyme cleavage of this duplex has established that the viral genome is not circularly permuted²⁰. The sizes of the single-stranded loops (B and F) are not known exactly, but they are less than 100 bases long, since they cannot be detected by electron microscopy. The observation that the loop F in the MVM duplex synthesised *in vitro* is extremely resistant to cleavage by S1 nuclease (D. C. W. unpublished results) suggests that this loop may be very small.

The structure determined for MVM DNA as shown in Fig. 1 has been demonstrated for several parvovirus genomes (M. B. Chow and D. C. W., to be published), and leads us to propose a model for the replication of such molecules. We have called this scheme the rolling hairpin as it is quasicircular and, in some respects, resembles the rolling circle model proposed for coliphage ΦX174 DNA replication by Gilbert and Dressler²⁴.

Rolling hairpin

The first step proposed for parvovirus DNA replication, shown in Fig. 2, is the gap-fill synthesis of the complement d of sequence D; as mentioned above, this step can be performed *in vitro* by several DNA polymerases²⁰. This is followed by displacement synthesis and gap-fill synthesis, to copy the 5' terminal hairpin sequence aBA. To continue synthesis, we propose that this terminal duplex palindrome is now rearranged to form the 'rabbit-eared' structure shown in Fig. 2iv. This terminal synthesis and rearrangement recreates the hairpin originally present at the 5' end of the parental genome. At the

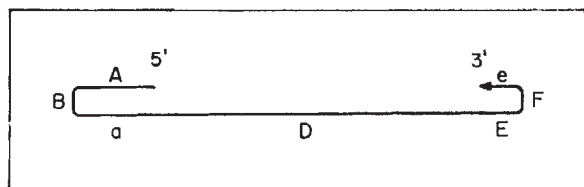


Fig. 1 Structure of MVM genome. In this and subsequent Figs, letters are used to represent unique blocks of sequence. The upper and lower cases denote complementary sequences, that is, if A is 5'-A-T-G-G-C-A-C-3' then a would be 5'-G-T-G-C-C-A-T-3'. The arrow denotes the 3'-OH end of a strand.

same time, it creates a copy of this hairpin, on the 3' end of the complementary strand, which can serve as a primer for the synthesis of the progeny strand. The new 3' hairpin eventually becomes the 5' hairpin of that future progeny genome, as shown in Fig. 2v. This process of 'hairpin transfer' is the essence of the model. The dimer length duplex is then completed by displacement synthesis. The resulting molecule comprises a single polynucleotide chain from which two viral genomes could be generated by suitable endonucleases; that is, the scheme shown in Fig. 2 represents one genome duplication. In the absence of endonuclease cleavage, however, this structure is obviously capable of continued synthesis by the same mechanism, yielding larger and larger multimers. In this respect our model strongly resembles the rolling circle model²⁴. Thus a further roll around the 5' hairpin (that is, a repeat of steps ii to vi in Fig. 2) would generate the tetramer as shown in Fig. 3. In the concatemers formed by this replication process, the viral genomes are situated alternately on opposite strands of the duplex, with their palindromic ends overlapping.

If the single-stranded loop sequences B and F are themselves palindromes, with a phosphodiester bond as the axis of

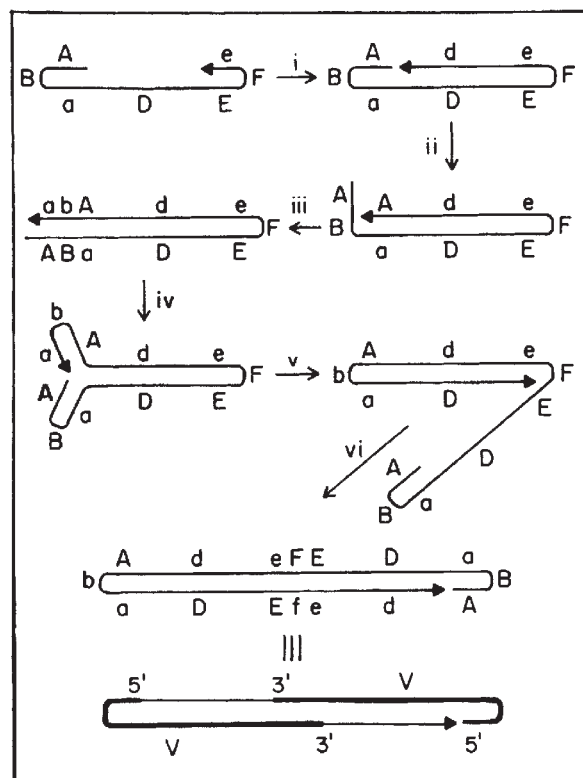


Fig. 2 The rolling hairpin model for parvovirus replicative DNA synthesis. i, Gap-fill synthesis; ii, displacement synthesis and iii, gap-fill synthesis to complete genomic hairpin; iv, enzymatic rearrangement of terminal palindrome to give rabbit-eared structure; v, displacement synthesis and vi, gap-fill synthesis. The molecule is now essentially the same structure as the first intermediate between steps (i) and (ii), and repetition of steps (ii) and (iii) generates the dimer genomic hairpin. Repetition of steps (iv), (v) and (vi) generate the tetramer, and so on for larger multimers.

rotational symmetry (for example, GGTACC), there would be no difference between sequences B and b or between sequences F and f, and all genomes would be exactly the same; if they are not palindromes, then there would be four different kinds of viral sequence which differed in two small regions.

So far we have described a scheme for generating and replicating duplex forms of parvovirus DNA, based on the structure of the genome. The model now allows an explanation of some hitherto puzzling physical properties of parvovirus replicative intermediates. We showed previously¹⁰ that some of the MVM duplex DNA molecules found in infected cells seemed to be concatemers up to about ten genome equivalents long. These molecules, however, contained no single strands longer than two genome equivalents, and on denaturation and reannealing formed predominantly monomer length duplexes. The long duplexes were also reduced to approximately monomer length by heating to submelting temperature. In addition we found that a large fraction of the MVM double-stranded DNA reannealed in a monomolecular fashion after denaturation, suggesting that viral and complementary strands were linked covalently, as predicted by the rolling hairpin model. Similar spontaneous renaturation behaviour has since been reported for duplex intermediates of parvovirus X 14 (ref. 14), AAV-2 (ref. 15) and Kilham rat virus¹⁸. Recently Straus *et al.*¹⁶, have reported the isolation, from AAV-infected cells, of dimer length duplexes which renature spontaneously. These comprise a single DNA chain, four genomes long, containing alternating viral plus and minus strands. They have proposed a model for the replication of AAV DNA involving hairpin primers which is similar in parts to that proposed in Fig. 2. We originally suggested¹⁰ that hairpin duplexes of genome length were formed by the use of the 3' end of the viral strand as a primer for the

synthesis of its complementary strand, and that this implied a mechanism for the regeneration of the primer in order to preserve the entire base sequence of the genome during replication. In the rolling hairpin model, this is accomplished by the process of hairpin transfer.

The overall physical properties of double-stranded DNA molecules replicating according to the model, such as the lengths of the single strands which the duplexes contain and the proportion of spontaneously renaturing molecules, will depend on how the intermediates are further processed to generate progeny genomes.

Synthesis and packaging of progeny viral DNA

A replication scheme which generates concatemers as replicative intermediates must necessarily include some process for excising progeny viral DNA. In parvovirus replication it is unlikely that this process relies only on the selection of genome size segments, since the viral DNA is not circularly permuted^{20,25}. We envisage the first step in the process to be the introduction of a single-strand break, at the 5' end of a genome within the

concatemer, that is between d and A in Fig. 3i. The 3'-OH terminus at this nick is then used as a primer for displacement synthesis of progeny DNA strands, giving rise to the duplex molecules with single-stranded side chains that are observed in infected cells^{10,12,17}. In addition, we suggest that this displacement synthesis is driven by concomitant packaging of the progeny strand because little, if any, free single-stranded DNA can be detected during infection^{13,14,17,18}. This idea is supported by the report²⁶ that a temperature-sensitive mutant of H-1 virus, with a virion which is physically unstable at high temperature, makes replicative intermediate, but not progeny DNA, at the non-permissive temperature.

After the 3' terminal sequence e has been displaced, excision of the progeny genome could be completed by another sequence-specific nuclease, perhaps an integral part of the virion, releasing an intact virus particle and stopping the displacement synthesis. This second nuclease must be inactive on double-stranded DNA, because otherwise it would also introduce a nick in the template strand. The nascent 3' OH terminal sequence e must be rapidly rejoined to the existing sequence d because the intact strand may be required as a template for the 3' terminus of another genome being synthesised in the opposite direction, as in Fig. 3iv. If the packaging of progeny strands drives the displacement synthesis, the continued synthesis of complementary, minus strands (sequence d) is avoided, and the asymmetrical synthesis of progeny genomes, which has been reported for the autonomous parvoviruses^{10,13,17} is assured. The specificity of such a packaging mechanism implies recognition of the 5' end of the genome by the precursor virion. It is interesting that when infectious particles of MVM are lysed gently and examined in the electron microscope, a significant number of capsids remains associated with an end of the extruded DNA²⁰. There is no detectable relationship between the two ends of MVM DNA²⁰, whereas the ends of most AAV genomes are related by an inverted terminal redundancy^{27,28}. If terminal sequence recognition by the particle controls strand selection, this could explain why MVM only packages the plus strand, whereas AAV packages both plus and minus strands.

In concatemers where the 5' ends of overlapping genomes have been nicked, displacement synthesis at one or both nicks will break the molecule in this region. Subsequent synthesis would regenerate the terminal palindrome of each of the fragments, which would then be able to continue replication after rearrangement as described in Fig. 2iv. Since none of the strands in double-stranded MVM DNA was found to be greater than dimer length¹⁰, we suggest that the duplex concatemers had been nicked, perhaps during isolation, at the 5' end of every component genome. Each continuous strand in such a molecule would have the sequence ABADEFed and therefore comprise two palindromes. These sequences could rearrange, through cruciform intermediates, to produce monomer length hairpin duplexes at elevated temperature. This could explain the breakdown of MVM concatemers to monomers when they are heated to subdenaturing temperatures¹⁰. As both palindromic sequences in the concatemer would, in effect, become terminal hairpins in the monomer, one would expect this process to be essentially irreversible.

Hairpin transfer process

The central process of the rolling hairpin model is the synthesis and rearrangement of the palindromic terminus, to produce a 'rabbit-eared' structure. This permits the direction of chain growth to be reversed with respect to the original duplex. The rearrangement need not require complete melting out of the terminal region. If the loop sequence B is short it would be possible to interconvert the two structures through a cruciform intermediate, originally described by Platt²⁹ as twist transfer. Maniloff³⁰ has suggested, on theoretical grounds, that such a rearrangement should exhibit a transitional energy barrier and would be unlikely to occur spontaneously in physiological conditions. Therefore we have to postulate that there is an

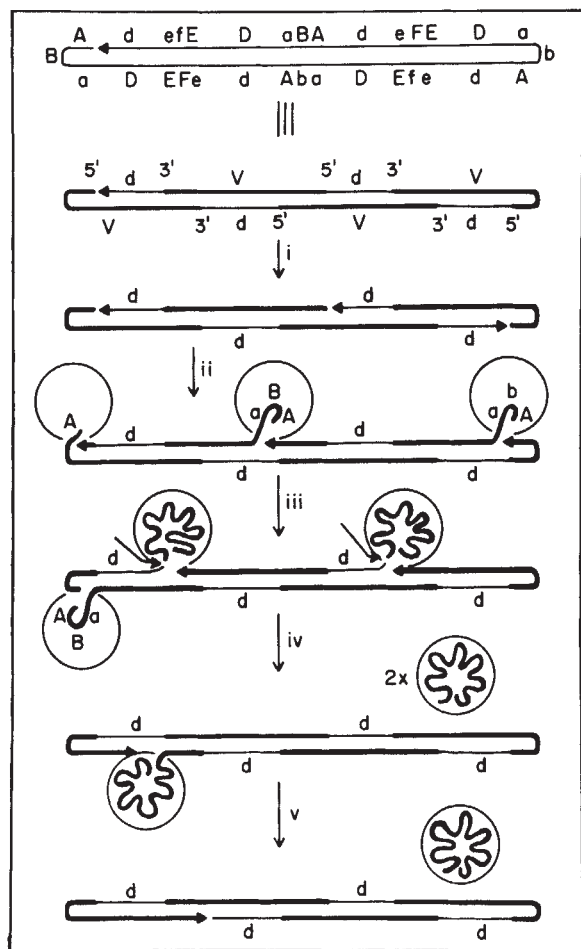


Fig. 3 Proposed mechanism for parvovirus progeny DNA synthesis, showing a tetramer intermediate. The progeny DNA strand, V, is indicated throughout as the heavy line. d represents the ~4,100 nucleotides of the complementary strand which is not packaged by the autonomous parvoviruses. The circle represents the capsid of an immature virion. *i*, Internal initiation of progeny strand displacement by sequence-specific nuclease cleavage; *ii*, displacement of progeny strand by elongation using the 3'-OH of the 'nick' as primer, and driven by concomitant packaging; *iii*, packaging of complete genome, and cleavage of 3'-OH end of genome (arrow); *iv*, release of intact virion and repair of the 3' end of newly synthesised progeny strand to the 5' end of its adjoining complementary strand; *v*, completion of a mature virion in the opposite direction and regeneration of the initial tetramer.

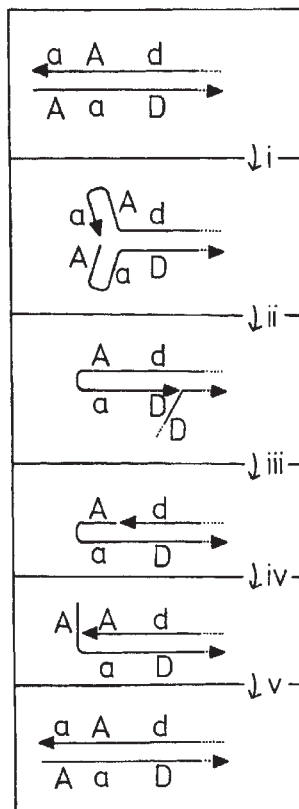


Fig. 4 Proposed model for eukaryote chromosome terminal replication. *i*, Enzymatic rearrangement of terminal palindrome; *ii*, strand-displacement synthesis of daughter strand using 3'-OH of the parental strand as primer; *iii*, sequence-specific nuclease cleavage followed by *iv*, strand displacement and *v*, gap-fill synthesis to regenerate terminal palindrome.

enzyme activity which catalyses the rearrangement, and suggest that it is part of the DNA replication system of the cell which parvoviruses and possibly other viruses, require for rearrangement of their chromosomal ends during replication.

Hairpin transfer and host cell replication

To overcome the problem of replicating termini, originally discussed by Watson⁴, Cavalier-Smith suggested⁵ that the ends of linear chromosomes are palindromic so that, after excision of the 5'-terminal RNA primer, the exposed DNA strand could form a 3'-OH terminated hairpin. He proposed that this is then joined to the 5' end of the complementary strand by gap-fill synthesis and ligation. The palindromic sequence is then nicked at its 5' end by a sequence-specific nuclease. A combination of strand displacement and gap-fill synthesis, using the 3'-OH of the nick as primer, reconstitutes the parental strand.

Based on studies with MVM we have proposed a model for the replication of the parvovirus genome which overcomes the problem of the completion of linear termini by the process we have described as hairpin transfer. This concept can be extended to the replication of host chromosomes and other linear DNA molecules, and thus becomes a modification of Cavalier-Smith's model, involving fewer separate steps. In this scheme (Fig. 4), the steps of strand separation, RNA primer synthesis, excision, hairpin formation, repair synthesis and ligation, are replaced by the enzymatic rearrangement of the terminal palindrome. This produces a 'rabbit-eared' structure whose 3' end acts as a primer for the synthesis of the complementary strand. A sequence-specific nuclease then nicks at the 5' end of the palindrome, and synthesis using the 3'-OH of the nick as primer, reconstitutes the terminal sequence as before.

It is reasonable to expect that the expression of an enzyme system responsible for the initiation and completion of replication at chromosome termini would be coordinated with the passage of the cell through the mitotic cycle; in this respect it is important that the autonomous parvoviruses depend on, but cannot induce some host function(s) expressed transiently in the S phase⁸. The limited genetic capacity of these viruses suggests that they alter the host cell replication machinery very little. We suggest that the parvoviruses usurp the host's system for duplicating the termini of its chromosomes, to replicate their own DNA by the rolling hairpin process.

Terminal palindromes, which form terminal hairpins on strand separation, are emerging as a common feature of linear chromosomes. In addition to MVM DNA²⁰, such sequence arrangements have been demonstrated in the genomes of AAV³¹ and herpesvirus³². The covalently continuous structure demonstrated for the vaccinia virus genome³³ could be regarded as a special form of terminal palindrome. These palindromes are found irrespective of the existence or polarity of terminally redundant regions in such molecules, either inverted as in adenovirus DNA^{34,35}, non-inverted as in herpesvirus DNA^{32,36}, or a mixture of both as found for populations of AAV DNA molecules^{9,27,28,37}.

Studies showing that a combination of exonuclease, polymerase and ligase can cross link the ends of coliphage T7 DNA^{38,39} indicate that the T7 genome similarly contains a terminal palindrome, which agrees with the observation⁴⁰ that the termini of T7 DNA contain a self-complementary sequence. The isolation, from T7-infected cells, of spontaneously re-naturing intermediates of greater than genome length⁴¹ suggests that this sequence might be used for the replication of T7 termini also. Terminal palindromes may, therefore, be necessary for the replication of all linear chromosomes which do not, like phages λ or T4, replicate by becoming circular.

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Relationships between structure and activity of retinoids

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Structure-function relationships of 19 retinoids were studied in three in vitro systems in defined, serum-free medium. These systems measure effects on differentiation and growth, as well as toxic effects. The ring, side chain, and polar terminal group of natural retinoids all may be modified synthetically to yield highly active compounds. Several retinoid ethers were found to be significantly less toxic than the corresponding carboxylic acids or alcohols.

RETINOIDS (the set of molecules comprised of vitamin A and its synthetic analogues) are known to exert profound effects in controlling cellular differentiation in epithelium of many target tissues, including bronchi and trachea, uterus, prostate, kidney and bladder, stomach, intestine, pancreatic ducts, and skin¹. It has recently been shown that several synthetic retinoids can prevent the development of epithelial cancer of the skin^{2,3}, respiratory tract⁴, mammary gland^{5,6}, and urinary bladder³⁸ in experimental animals. In these experiments, the retinoids were not given to the animals until after completion of administration of the respective carcinogens. In these conditions, the retinoids do not prevent initiation of carcinogenesis, but rather, they modify preneoplastic states during the latent period of cancer development, before the onset of invasive malignancy. Although retinoids may act by more than one mechanism to modify preneoplastic states, their direct ability to control cell differentiation in target epithelia is undoubtedly of major importance in their suppression of carcinogenesis. For example, it has been shown that direct application of retinoids to prostatic^{7,8} and tracheal⁹ organ cultures can reverse hyperplastic or anaplastic lesions induced by chemical carcinogens; in these experiments, also, retinoids were not added to the cultures until after the lesions had been initiated by the carcinogen.

Many synthetic retinoids have now been made, in which there has been substantial modification of the ring, side chain, or polar terminal group of the natural vitamin A molecule. For both practical and theoretical considerations, an analysis of structure-activity relationships in this set of molecules is of fundamental importance. It is important to establish not only the molecular parameters that are required for retinoids to control epithelial cell differentiation and to prevent carcinogenesis, but also to establish the molecular parameters responsible for the undesirable toxic effects which retinoids may have on tissues, since such toxic effects may limit the effective use of retinoids in cancer prevention¹⁰. It is already clear that several synthetic retinoids, which have potent activity for the prevention of cancer in experimental animals, for the control of epithelial cell differentiation *in vitro*, or for reversal of premalignant lesions *in vitro*, have little capacity to promote growth in animals maintained on vitamin A-deficient diets¹¹. Thus, classical nutritional methods (promotion of growth in vitamin A-deficient animals) may not be particularly useful to assess the potential activity of retinoids in cancer prevention, and new methods must be developed for correlation of structure and activity. Although measurement of ability to prevent various types of epithelial cancer in the intact experimental

animal is an important measure, such tests are very expensive and time-consuming when assaying large numbers of new compounds. Moreover, it is difficult to evaluate cellular and biochemical mechanisms of action in the live animal.

For these reasons, we have assayed the activity of retinoids in several organ and cell culture systems, using chemically defined, serum-free medium, in which structure-activity relationships can be evaluated quantitatively. Here we report on the biological activity of 19 retinoids in three such *in vitro* systems, which measure effects on both cell differentiation and growth (reversal of keratinisation in retinoid-deficient tracheal epithelium, increase of RNA in epidermal cell cultures), as well as the toxic effects on tissue (destruction of tracheal cartilage). More than 5,500 organ and cell cultures (including untreated control cultures) were used in these assays. Finally, since it is important that *in vitro* measurements be extended to evaluate utility *in vivo*, we also describe some major *in vivo* changes in tissue distribution of retinoids which result from synthetic modification of the polar terminal group.

Methods for *in vitro* studies

Structures of the retinoids (all provided by Hoffmann-La Roche Inc., Nutley, New Jersey and F. Hoffmann-La Roche & Co. A. G., Basel, Switzerland) used in the present study are shown in Fig. 1 and Table 1. Three series of retinoids have been studied: Fig. 1a retinoids which retain the β -cyclohexenyl ring and the all-*trans* side chain of the natural retinoids, but which have modifications of the polar terminal group; Fig. 1b retinoids in which a fluorine atom has been inserted into the all-*trans* side chain; and Fig. 1c retinoids which have a substituted

Fig. 1 Structures of retinoids in Table 1.

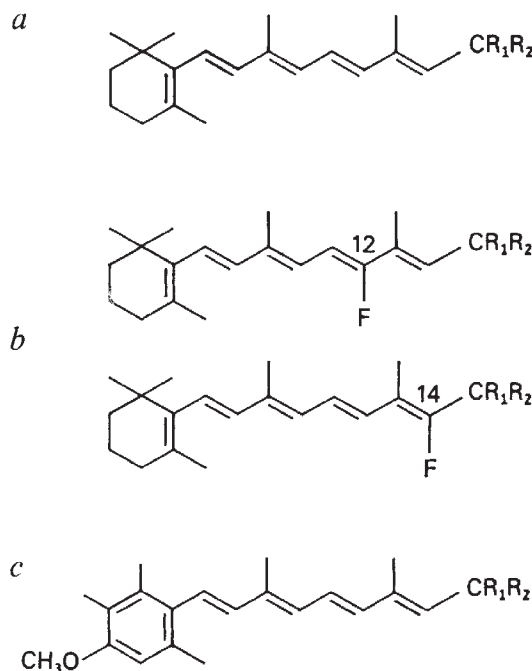


Table 1 Activity of retinoids in tracheal organ cultures and epidermal cell cultures

Structure, R ₁ =, R ₂ =	Trivial name	Reference	Reversal of keratinisation, tracheal organ culture, ED ₅₀ (M)	Increase in RNA, epidermal cell culture, ED ₅₀ (M)	Toxicity to cartilage, tracheal organ culture, TD ₂₀ (M)
Fig. 1a, -H ₂ , -OH	Retinol	26	2 × 10 ⁻⁹ (54)*	9 × 10 ⁻¹⁰ (64)	2 × 10 ⁻⁶ (67)
Fig. 1a, -H ₂ , -OCH ₃	Retinyl methyl ether	20, 27	3 × 10 ⁻⁹ (122)	4 × 10 ⁻⁹ (83)	1 × 10 ⁻⁵ (103)
Fig. 1a, -H ₂ , -OC ₄ H ₉	Retinyl butyl ether	20	3 × 10 ⁻⁸ (55)	> 1 × 10 ⁻⁶ (40)	not toxic at 1 × 10 ⁻⁵ (42)
Fig. 1a, -H ₂ , -OCOCH ₃	Retinyl acetate	26	3 × 10 ⁻⁹ (123)	1 × 10 ⁻⁹ (18)	6 × 10 ⁻⁶ (63)
Fig. 1a, -H ₂ , -NHCOCH ₃	N-Acetyl retinyl amine	28	9 × 10 ⁻⁹ (113)	> 1 × 10 ⁻⁶ (52)	not toxic at 1 × 10 ⁻⁵ (50)
Fig. 1a, -H ₂ , -N(CO) ₂ C ₆ H ₄	N-Retinyl phthalimide	28	5 × 10 ⁻⁷ (49)	inactive at 1 × 10 ⁻⁶ (48)	not toxic at 1 × 10 ⁻⁵ (10)
Fig. 1a, -H ₂ , =O	Retinal	26	3 × 10 ⁻¹⁰ (98)	8 × 10 ⁻¹⁰ (37)	5 × 10 ⁻⁷ (49)
Fig. 1a, -H ₂ , =NNHCOCH ₃	Retinal acetylhydrazone	29	4 × 10 ⁻¹⁰ (54)	3 × 10 ⁻⁹ (35)	7 × 10 ⁻⁷ (25)
Fig. 1a, -H ₂ , =NOH	Retinal oxime	30	1 × 10 ⁻⁸ (61)	9 × 10 ⁻⁹ (31)	2 × 10 ⁻⁶ (25)
Fig. 1a, =O, -OH	Retinoic acid	26	3 × 10 ⁻¹⁰ (337)	4 × 10 ⁻¹⁰ (287)	1 × 10 ⁻⁷ (116)
Fig. 1a, =O, -OC ₂ H ₅	Retinoic acid ethyl ester	31	5 × 10 ⁻¹⁰ (119)	6 × 10 ⁻⁹ (100)	2 × 10 ⁻⁷ (18)
Fig. 1a, =O, -NHC ₂ H ₅	Retinoic acid ethyl amide	32	2 × 10 ⁻⁹ (52)	> 1 × 10 ⁻⁶ (70)	9 × 10 ⁻⁷ (32)
Fig. 1b, 12-fluoro =O, -OC ₂ H ₅	12-Fluororetinoic acid ethyl ester	33	3 × 10 ⁻¹⁰ (63)	1 × 10 ⁻⁷ (40)	2 × 10 ⁻⁷ (18)
Fig. 1b, 14-fluoro =O, -OC ₂ H ₅	14-Fluororetinoic acid ethyl ester	34	4 × 10 ⁻¹⁰ (68)	1 × 10 ⁻⁸ (40)	5 × 10 ⁻⁸ (18)
Fig. 1c, -H ₂ , -OH	TMMP analogue of retinol	35	2 × 10 ⁻⁸ (48)	9 × 10 ⁻⁸ (39)	9 × 10 ⁻⁷ (34)
Fig. 1c, -H ₂ , -OCH ₃	TMMP analogue of retinyl methyl ether	35	3 × 10 ⁻⁸ (70)	5 × 10 ⁻⁷ (38)	4 × 10 ⁻⁶ (57)
Fig. 1c, =O, -OH	TMMP analogue of retinoic acid	35	6 × 10 ⁻⁹ (187)	7 × 10 ⁻⁹ (86)	6 × 10 ⁻⁸ (88)
Fig. 1c, =O, -OC ₂ H ₅	TMMP analogue of retinoic acid ethyl ester	35	2 × 10 ⁻⁸ (53)	2 × 10 ⁻⁷ (31)	2 × 10 ⁻⁷ (63)
Fig. 1c, =O, -NHC ₂ H ₅	TMMP analogue of retinoic acid ethyl amide	35	3 × 10 ⁻⁸ (66)	5 × 10 ⁻⁸ (32)	1 × 10 ⁻⁶ (52)

ED₅₀(M) for reversal of keratinisation in tracheal organ culture is the dose for reversal of keratinisation in epithelium of 50% of retinoid-deficient hamster tracheas using standard assay conditions¹³. ED₅₀(M) for increase in RNA in epidermal cell culture is the dose for 50% increase in total RNA in mouse epidermal cells, also using standard assay conditions¹⁴. TD₂₀(M) for toxicity to tracheal organ culture is the dose for release of 20% of total proteoglycan from hamster tracheal cartilage into culture medium using standard assay conditions (see text).

*Numbers of organ or cell cultures used for evaluating each retinoid are shown in parentheses.

aromatic (trimethylmethoxyphenyl, TMMP) ring, as well as modifications of the polar terminal group. All retinoids were dissolved in dimethylsulphoxide (DMSO) before addition to cultures; an equivalent amount of DMSO (which never exceeded 1%) was added to all control cultures.

The test systems both for assay of reversal of keratinisation in organ cultures of tracheal epithelium from retinoid-deficient hamsters^{12,13}, and for assay of increase in RNA in cell cultures of mouse epidermis¹⁴, have been described in detail. Typical dose-response curves for effects of retinoids in reversing keratinisation in tracheal organ culture are shown in Fig. 2; typical dose-response curves of their effects in increasing RNA in epidermal cell cultures have been published previously¹³. Toxicity of retinoids to cartilage of hamster trachea in organ culture was measured by a modification of a procedure for assay of retinoid toxicity to rabbit ear cartilage¹¹. Briefly, tracheae from 4-5-week-old hamsters fed a normal laboratory stock diet were removed in sterile conditions, trimmed carefully of connective tissue, and then cultured for 3 d in the same medium used for the assay of keratinisation reversal, using air containing 5% CO₂ as the gas phase. These tracheal organ

cultures were exposed to retinoids during the entire 3-d period. Crystalline bovine serum albumin (0.5%) was added to some cultures to ensure solubility of retinoids at the highest concentrations used. After this period, both the trachea and the culture medium from each dish were saved; the amount of cartilage proteoglycan released into the culture medium, as well as that remaining in the cartilage, was measured with Alcian blue¹⁵. The total proteoglycan remaining in the cartilage was solubilised by papain digestion (digestions were performed at 37 °C for 2 h with shaking, using crystalline papain, 1 mg ml⁻¹, in 0.05 M sodium acetate buffer, pH 5.8, containing 0.001 M cysteine HCl). Chondroitin sulphate was used as the proteoglycan standard. At the end of the assay, the amount of proteoglycan found in the culture medium and the amount remaining in the cartilage were summed, and toxicity for any retinoid at any dose level was plotted (Fig. 3) as the percentage of the total proteoglycan released into the medium¹¹.

Reversal of keratinisation by retinoids

The ability of the respective retinoids to suppress keratinisation and reverse squamous metaplastic lesions caused by retinoid

Table 2 Comparison of tissue levels of retinoids 24 h after either a single or the last of nine oral doses

Retinoid	Single dose			Nine doses		
	Number of rats	Liver	Breast	Number of rats	Liver	Breast
Solvent control	18	0.37±0.02	0.0052±0.0003	19	0.53±0.02	0.0088±0.0015
Retinyl acetate	18	1.82±0.13	0.014±0.002	17	11.80±0.75	0.082±0.004
Retinyl methyl ether	17	0.92±0.10	0.060±0.01	10	7.38±0.98	0.71±0.10
Retinyl butyl ether	12	0.45±0.04	0.026±0.004	12	0.79±0.03	0.10±0.01

Sprague-Dawley female rats were dosed orally with retinoids (30 µmol each dose) dissolved in 0.4 ml of ethanol-trioctanoil (1:3). The rats receiving nine doses were treated three times weekly. All rats were killed 24 h after their final dose and tissue levels were determined with the trifluoroacetic acid method³⁷ after extraction of tissue with ethyl ether³⁶. Retinyl acetate, retinyl methyl ether, and retinyl butyl ether all gave linear standard reactions with trifluoroacetic acid at 616 nm. Figures are total µmol of retinoid found per g wet weight tissue ± standard error.

deficiency in tracheal epithelium is shown in Table 1 and Fig. 2. This assay is a very sensitive measure of the ability of retinoids to control normal epithelial cell differentiation, since the same process can be shown to occur in the live animal¹. Dose-response plots (Fig. 2) were made for all retinoids, and the values derived for the dose effective in suppressing keratinisation in half the cultures are shown in Table 1. Many retinoids were extremely active in the 10⁻⁹ M range (approximately 300 pg ml⁻¹).

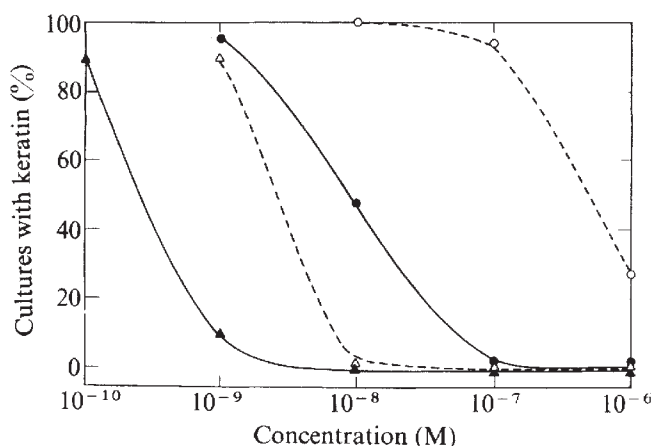


Fig. 2 Dose-response curves for reversal of keratinisation in organ cultures of retinoid-deficient tracheal epithelium by application of retinoids. Tracheae (one per culture dish) were treated with retinoids for 7 d before scoring for the presence of both keratin and keratohyaline granules. ▲, Retinoic acid; △, retinyl acetate; ●, *N*-acetyl retinyl amine; ○, *N*-retinyl phthalimide. Similar curves were obtained for all retinoids reported in Table 1, and the ED₅₀ was derived from these plots. Numbers of tracheae used in these experiments are shown in Table 1.

Several interesting results can be seen. In both series of retinoids—those with a β-cyclohexenyl ring (Fig. 1a), as well as those with an aromatic TMMP ring (Fig. 1c)—compounds with a carboxylic acid terminus were slightly more active than those with a carboxylic acid ethyl ester or carboxylic acid ethyl amide terminus, although the differences were not great. Also, in both series, compounds with terminal methyl ether functions were almost as active as the corresponding alcohols. Retinyl butyl ether was one-tenth as active as retinyl methyl ether or retinyl acetate. The oxygen atom on the terminal carbon of retinyl acetate may be replaced with an amine function, resulting in the compound *N*-acetyl retinyl amine, which was one-third as active as retinyl acetate. The *N*-phthalimide derivative was essentially inactive, except at the highest concentration tested. The acetylhydrazone derivative of retinal was almost as active as the parent aldehyde, although the oxime derivative of retinal had much less activity. The 12- and 14-fluoro-derivatives of retinoic acid ethyl ester were very active in reversing keratinisation.

RNA increase in epidermal cultures by retinoids

The ability of the respective retinoids to increase RNA in epidermal cell cultures is also shown in Table 1. There is a remarkable correspondence between activities as measured in the tracheal organ culture assay and the epidermal cell culture assay for 15 of the 19 retinoids. Three retinoids (retinyl butyl ether, *N*-acetyl retinyl amine, and retinoic acid ethyl amide) which reverse keratinisation in tracheal epithelium, were essentially inactive in the epidermal cell culture system, and a fourth compound, 12-fluororetinoic acid ethyl ester, was markedly less active in the epidermal cell assay. The reason for the above four exceptions is not known; but it is conceivable that epidermal cells from newborn mouse skin are deficient in the appropriate enzymes which might activate these four retinoids to biologically active forms in tracheal epithelium.

Toxicity of retinoids to tracheal cartilage

The toxicity of retinoids to tracheal cartilage *in vitro* is shown in Table 1 and Fig. 3. Dose-response plots (Fig. 3) were made for all retinoids, and the values derived for the dose that would release 20% of the total tracheal proteoglycan are shown in Table 1. Retinoic acid, retinoic acid ethyl ester, 12- and 14-fluororetinoic acid ethyl ester, the TMMP analogue of retinoic acid, and the TMMP analogue of retinoic acid ethyl ester were the six most toxic compounds. In both series of retinoids (Fig. 1a, c), the ethyl amide derivatives were markedly less toxic than their respective parent carboxylic acids. Similarly, the methyl ether derivatives were significantly less toxic than their respective parent alcohols, which in turn, were less toxic than the corresponding carboxylic acids. Two compounds, namely retinyl butyl ether and *N*-acetyl retinyl amine, which could control tracheal epithelial cell differentiation *in vitro* were virtually without toxicity to tracheal cartilage *in vitro*. These two compounds were also essentially inactive in the epidermal cell culture assay. All these data suggest that metabolic activation of retinyl butyl ether and *N*-acetyl retinyl amine may be required for their activity, and that, in some circumstances, there may even be a different set of metabolites which control cellular differentiation and cause cellular toxicity.

Pharmacokinetics of retinoids

The above *in vitro* studies clearly show a wide range of changes in activity resulting from modification of the structure of the retinoid molecule. Practical usefulness of retinoids for cancer prevention depends not only on a compound having the desired activity and lack of toxicity, but also on its pharmacokinetics *in vivo*. Thus, although retinyl acetate is highly active and relatively non-toxic (compared with retinoic acid) *in vitro*, it has the undesirable property *in vivo* of being stored in the liver, mostly as retinyl palmitate^{16,17}. These elevated levels of retinyl palmitate can eventually cause serious toxic effects, both in the liver and systemically¹⁷. Moreover, because of the tendency of retinol and its esters to be stored in the liver, these retinoids may not reach specific target organs in doses as high as might be desired.

The greater effectiveness of retinyl methyl ether, compared with retinyl acetate, in prevention of mammary cancer in rats has recently been reported⁶. One possible explanation of these findings might be that greater effective levels of retinyl methyl ether than of retinyl acetate were attained in the mammary gland, so we carried out experiments to investigate this possibility. High doses of retinoids, corresponding to those used previously⁶ when prevention of mammary cancer was investigated, were given in these experiments. After either single or multiple oral dosing of retinyl methyl ether, much greater levels of this compound (or one of its metabolites which react positively with trifluoroacetic acid, TFA) were indeed found in the mammary gland and adjacent fat pad, than after similar dosing with retinyl acetate (Table 2). Retinyl methyl ether *in vivo*, however, is known to be cleaved to retinol and then to be stored in the liver as retinyl ester^{18,19}; results shown in Table 2 confirm this known ability of the liver to store large amounts of retinyl methyl ether or its metabolites. Thus, although retinyl methyl ether yields high retinoid levels in the mammary gland and adjacent fat pad, the storage of this compound and its metabolites in the liver suggests that, if retinyl methyl ether is fed for long periods, it may be as potentially toxic to the liver as retinol or retinyl esters.

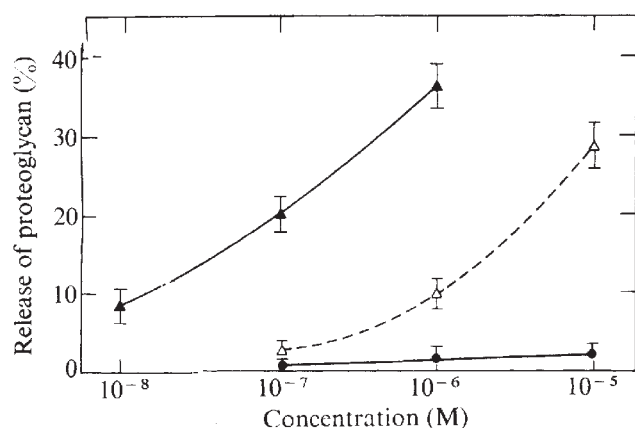


Fig. 3 Dose-response curves for toxic effects of retinoids to tracheal cartilage in organ culture. Tracheae (one per culture dish) were treated with retinoids for 3 d before measuring both proteoglycan released into the culture medium, and that remaining in the cartilage. $\blacktriangle$, Retinoic acid; $\triangle$, retinyl acetate; $\bullet$, *N*-acetyl retinyl amine. s.e.m. of measurements is shown. Numbers of tracheae used in these experiments are shown in Table 1.

In contrast, another retinoid ether, retinyl butyl ether, seems to be metabolised by the intact animal in an entirely different way from retinyl methyl ether (Table 2). In spite of the fact that multiple dosing of retinyl butyl ether caused a twelvefold increase in the amount of TFA-positive retinoid in the breast, it did not even cause a twofold increase in liver retinoid in these experiments; in contrast, retinyl acetate and retinyl methyl ether respectively caused 22-fold and 1.4-fold increases in liver retinoid levels. The data indicate a major difference between the metabolism of retinyl butyl ether and of retinyl methyl ether, which we are now investigating using liver microsomes *in vitro*. Retinyl butyl ether thus seems to have the following unusual cluster of properties: it is a derivative of retinol with very low direct toxicity to tissue; it has significant growth-promoting activity in the whole animal²⁰; and it does not accumulate in the liver after repeated oral dosing at high levels.

Conclusions

The data presented above show the complexity of relationships between structure and activity in retinoids. Significant modifications have been made in all three regions (ring, side chain,

and polar terminus) of the retinoid molecule and these modifications have resulted in biologically active molecules that may have practical use. Although it would not be justified to generalise to other retinoids from the present data, the following conclusions may be drawn from the present studies on retinoids with β -cyclohexenyl and TMMP rings: (1) the presence of a terminal carboxylic acid function conveys high activity, but is also associated with high toxicity; (2) fluorine atoms may be inserted into the side chain of β -cyclohexenyl retinoids to yield highly active compounds, of which the pharmacokinetics are at present largely unknown; (3) a terminal amine function, as in *N*-acetyl retinyl amine, is compatible with activity and may result in an analogue with low direct toxicity to tissue; and (4) modification of the polar terminal group can markedly lessen toxicity, and as shown with retinyl methyl ether and retinyl butyl ether, may also usefully affect pharmacokinetic properties.

The present data yield little information about the 'active form' of retinoids. A variety of retinoid structures are now known to be active in control of cell differentiation. The recent description and purification of several retinoid-binding proteins²¹⁻²⁵, which may have some resemblance to steroid-binding proteins, suggest that there need not be a single active form of retinoids. Rather, a wide range of retinoid structures may interact with cellular receptors directly in a biologically significant manner.

We acknowledge our collaboration with Hoffmann-La Roche Inc., Nutley, New Jersey and F. Hoffmann-La Roche & Co., A. G., Basel, Switzerland, for both the retinoids used in this study, and also many useful discussions with members of their staff. We thank Eli Lilly and Upjohn for insulin and hydrocortisone hemisuccinate used in all organ cultures, and Drs John Bieri, DeWitt Goodman, P. M. Horsfield, Albert Kapikian, and Stuart Yuspa for valuable suggestions for the *in vitro* assays. Doris Overman and Joseph Smith provided technical assistance.

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letters to nature

Do superheavy elements imply the existence of black holes?

THE discovery¹ of the superheavy elements 116, 124, and 126 raises the question of where these elements are likely to have been formed. The majority of the post-iron-peak nuclei are thought to have been produced in conditions of explosive nucleosynthesis (the r-process), particularly in conventional supernova explosions. The ability of the r-process to produce superheavy elements is, however, very uncertain². The conditions necessary for superheavy element synthesis (β -decays occurring sufficiently slow that the $n \rightleftharpoons \gamma n$ equilibrium is not disturbed) are difficult to realise in astrophysical situations. The n-process (J. B. Blake and D. N. Schramm, unpublished) requires less extreme conditions (the β decays are important) and may occur more often. The majority of the elements normally attributed to the r-process may have been synthesised in this way. Neutron-induced fission causes both processes to terminate at nuclei with high proton numbers, Z , but the n-process may allow it to reach the higher Z value.

We note that superheavy elements, such as those discovered and others much less stable in our environment, must exist in the outer layers of a neutron star³. Moreover, ideal conditions for the production of superheavy nuclei (high neutron flux and rapid β decays) are found in the disruption of a neutron star. The β decay rate is then fast compared with the expansion time scale (ref. 4 and J. M. Lattimer and D. N. Schramm, unpublished). We envisage such disruption being possible in either of two ways, both of which involve a black hole. Lattimer and Schramm (unpublished) and Lattimer *et al.*⁴ have considered the tidal disruption of a neutron star from a close encounter with a black hole. Most of the disrupted star is swallowed by the hole, but some of the processed stellar material is assumed to escape.

Perhaps a more likely situation in which a neutron star is disrupted occurs when it accretes sufficient material that its mass exceeds the maximum mass for stable neutron stars. It has no alternative other than to collapse to form a black hole, and it again seems plausible that some of the outer layers are thrown off as it does so. The binary X-ray sources such as Her X-1 and Cen X-3 provide evidence that accreting neutron stars do exist.

The accretion process is enhanced if such binary systems tend to evolve towards coalescence, as is expected. One product of such evolution could be the giant stars envisaged by Thorne and Zytzkow⁵, in which accretion on to a neutron star core is a major power source for the whole star. Assuming that the neutron star cannot accept material at a rate much above that consistent with the Eddington limiting luminosity ($\sim 10^{38}$ erg s⁻¹), then $\sim 10^8$ yr are required before the collapse takes place. The resultant explosion could observationally closely resemble that of a more conventional supernova.

We thus argue that the most likely site for the production of superheavy elements is in the surface layers of a neutron star. The most plausible means by which these layers can be returned to the interstellar medium involve the intervention, or formation, of a black hole. We should, however, mention that in certain supernova models involving a hard equation of state, it is conceivable that the central regions collapse to neutron star

densities, form superheavy elements, and then bounce. This may disrupt the original star entirely, leaving no remnant at all.

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Do superheavies come from neutron stars?

THE recent report of discovery of superheavy elements¹ (with nuclear charges near the predicted values for shell closure, $Z = 114$ and $Z = 126$), in surprisingly large quantities in terrestrial material, raises the question of the astrophysical sources for such nuclei. The very lightest nuclei may have been created in the first hours of the big bang, but significant amounts of elements beyond helium could not have been formed in these conditions. The heavier elements must have been formed in stars, and those as massive as uranium were probably formed by intense neutron bombardment immediately before or accompanying supernova explosions². It seems unlikely, however, that superheavy nuclei could have been formed in this way, since attempts to reach the 'stability islands' by successive neutron captures must proceed by nuclei with very short lifetimes. This would almost certainly be the case for the island about $Z = 126$, if not for that at $Z = 114$. I would suggest that neutron stars might be a source for such superheavy nuclei.

Before theories of nucleosynthesis had reached their present state, Mayer and Teller considered evaporation from a fluid 'polyneutron' as a source of heavy elements³, and came to the conclusion that nuclei with atomic masses of several hundred might be formed. With some qualification, such a polyneutron may be identified with a neutron star. We now know that the surface of a neutron star will very quickly be cooled below the crystalline melting point by neutrino emission processes⁴, but it will be fluid for a short time after the collapse of a supernova core. Densities considerably higher than equilibrium values of perhaps $\rho \sim 10^{11}$ g cm⁻³ might be reached at the surface during oscillations following collapse. Evaporation of superheavies from the surface of such a newly formed neutron star would thus be virtually simultaneous with formation of heavy elements by neutron capture in the supernova explosion itself. Fission and β decay of the initial droplets of nuclear matter would be expected to yield some of the relatively stable elements about $Z = 114$, 126 and perhaps 164.

Another possibility is the disruption of a neutron star by tidal interaction with a black hole⁵. Matter can be ejected to infinity in such an encounter, and this matter might include

nuclear droplets large enough to give rise to the superheavy elements.

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Spatial and temporal variations of the atmospheric sodium layer observed with a steerable laser radar

LASER radar observations of resonance scattering from atmospheric sodium during the night have, on occasions, shown evidence of short term changes in the height distribution^{1–3}. It has been suggested that these changes arise from the movement of a horizontally structured sodium layer, but the measurements have all been limited to the vertical direction, and have provided no direct evidence of a horizontal variation. Evidence of horizontal structure at mesospheric heights has been provided by observations of hydroxyl emissions in the airglow, both from the ground⁴ and from an aircraft^{5,6}. In addition, the observed fluctuations of intensities and infrared rotational temperatures of the hydroxyl bands have been associated with the passage of atmospheric gravity waves^{7,8}. We report here observations intended to examine the short term and small scale horizontal variations of the sodium layer which might also be associated with such waves.

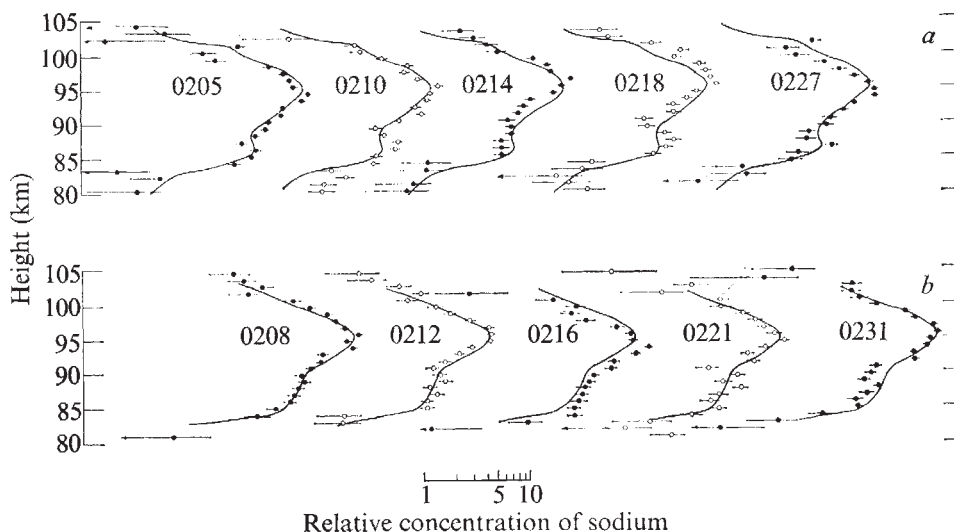
The measurements were carried out using a laser radar system incorporating a flashlamp-pumped dye laser and a steerable mirror used for both transmission and reception. The details of the system will be described elsewhere. The steerable mirror was generally alternated between fixed angular positions with runs of ~ 500 laser pulses for each position. By this means, measurements of the height distribution of sodium at various horizontal separations were obtained.

The measurements made with the laser beam directed alternately towards the zenith and 10°E during the early morning of September 21, 1975 are shown in Fig. 1. For both directions the measurements taken during each 2–3-min interval are shown together with the standard errors; these results have been derived from the photon counts for each 1-km height interval, after allowing for the background noise

and correcting for the range dependence. The curves show, for comparison, the mean height distributions derived for each direction separately, the density scale being adjusted to give the best fit to the individual measurements near the peak. No attempt has been made to express the results in absolute sodium concentrations because of the possible variability of the atmospheric transmission coefficient and uncertainties arising from aerosol scattering in a calibration procedure based on returns from lower heights. The results shown for the two directions in Fig. 1 provide evidence of short term changes in the sodium layer which are different at locations separated by as little as 15 km at the height of the layer. Specifically, the sequence of measurements for the zenith, in comparison with the mean curve, indicates an upward movement of the layer between 0210 and 0214 or 0218 and a return to below the original height by 0227. The corresponding change in the data for the 10°E direction is seen as a downward movement between 0212 and 0216; a return to the original height, and even an upward movement, is suggested in the data for 0231. These results imply vertical movements of ~ 2 km in the sodium layer, these movements being of opposite sense at points separated by ~ 15 km. Furthermore, the suggestion of a reversal in the sense of the movement near the end of the observing period for each direction is consistent with an oscillation of period ~ 20 min. This is the first time such a small scale horizontal structure has been detected in the atmospheric sodium layer. With the data available it is not possible to distinguish between a horizontally moving structure and the passage of an atmospheric gravity wave. We note, however, that a simple interpretation of such a periodic oscillation with a horizontal wavelength of ~ 30 km in terms of a gravity wave propagating in the absence of a horizontal wind would require a vertical wavelength of ~ 7 km, whereas the vertical motions seem to be cophasal over the whole weight range of the sodium layer.

Our programme of measurements, which were carried out over eight nights during the period September 1975–January 1976, showed that, in general, the sodium layer showed little small scale spatial variation or temporal changes over periods up to ~ 1 h. This is illustrated in Fig. 2 which shows the measurements carried out on October 5, 1975 with the laser beam directed 10°E and 10°W of the zenith, corresponding to a horizontal separation of ~ 30 km at the height of the layer. The results for each direction show no evidence of any pronounced variation of the layer over the 40-min observing period. Moreover, a detailed comparison of the data on which the mean curves for the two directions are based shows that the differences are not significant. On two of the nights of observation, beam inclinations of 30° to the zenith were used, corresponding to a horizontal separation of ~ 100 km at the height of the layer. Even with this separation, significant differences

Fig. 1 Variations of the height distribution of atmospheric sodium in the zenith (a) and at 10°E to the vertical (b), corresponding to a horizontal separation of ~ 15 km at a height of 85 km. The curves represent the mean of all observations shown at each angle. Data for September 21, 1975.



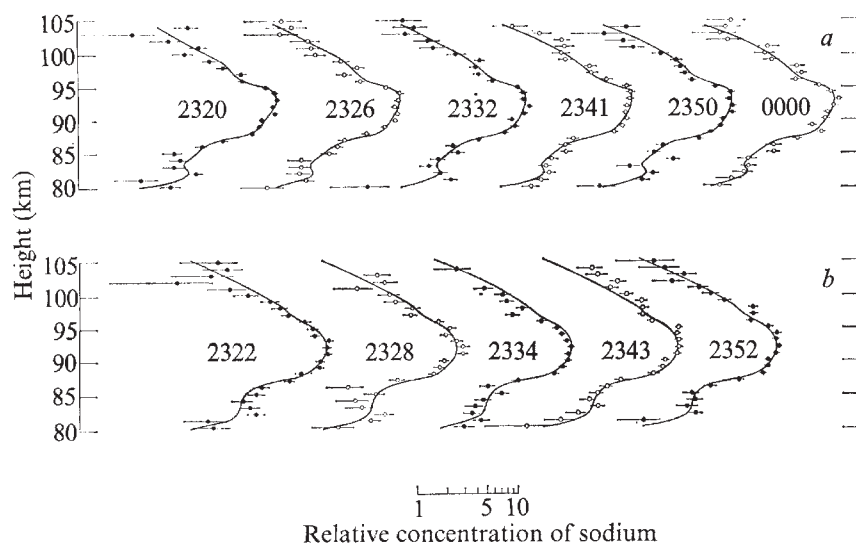


Fig. 2 Variations of the height distribution of atmospheric sodium at 10°E (a) and 10°W (b) to the vertical corresponding to a horizontal separation of ~ 30 km at a height of 85 km. The curves represent the mean of all observations shown at each angle. Data for October 5, 1975.

were observed only at heights < 82 km. In connection with this lack of variability, it should be noted that previous measurements at middle latitudes of the height variation of atmospheric temperature⁹ and of very low frequency waves reflected from the D region¹⁰ have indicated that the greatest degree of variability at mesospheric heights would be expected during winter months. It is intended to extend this type of measurement with improved system sensitivity and greater horizontal separation of the region being sounded.

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Atmospheric temperature calculated for ozone depletions

THE proposal that the Earth's protective ozone layer is being depleted by free chlorine produced by the photolytic decomposition of chlorofluoromethanes (CFC₁ and CFC₂, R11 and R12) has been the subject of much discussion and controversy^{1–3}. Although the effects of ozone removal on human health is a prime concern, attention has also been directed towards the possible influence on the stratospheric temperature profile⁴ with associated consequences for the climate. In previous papers^{4,5} it was shown that uniform depletion of stratospheric ozone leads to cooling of the stratosphere. To obtain a realistic bound on the maximum extent of stratospheric cooling, we have computed the thermal effects resulting from model D of Wofsy *et al.*⁵ for ozone depletion caused by chlorofluoromethane decomposition.

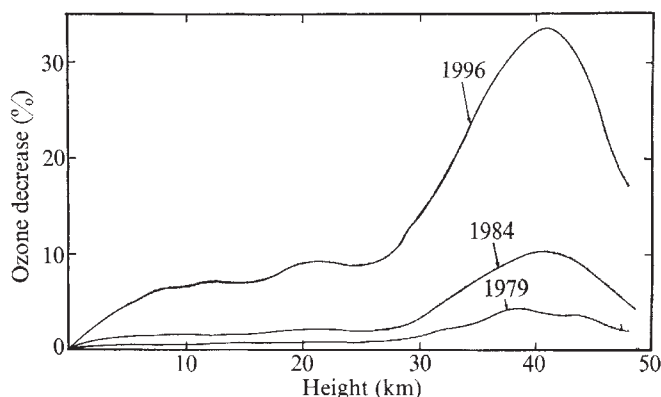
In model D the authors calculated the time dependence of the atmospheric ozone profile for an assumed 10% yr⁻¹ growth rate of R11 and R12 usage (equivalent to doubling every 7 yr), assuming the 1972 emissions were 2.2×10^5 and 3.5×10^5 tonnes respectively. The decrease from the 1972 ozone profiles are shown in Fig. 1 for the years 1979, 1984, and 1996 (the ozone profiles were obtained from S. C. Wofsy, private communication).

As an input to their calculations, Wofsy *et al.*⁵ used a temperature profile which was based on the 1966 standard atmosphere and assumed to remain unchanged through 1996. They report that their model gives a good representation of the ozone profile for the undisturbed atmosphere, when compared with available data⁷.

To establish internal consistency in our results, we first computed a 1972 temperature profile using the Manabe-Wetherald (M-W) model⁸, and the Wofsy *et al.* 1972 unperturbed ozone profile. The results were in essential agreement with Wofsy's assumed temperature profile. Next the 1979, 1984 and 1996 temperature profiles were computed assuming only that the ozone profiles changed in accordance with Wofsy's model D prediction (Fig. 1). The results are shown in Fig. 2.

As shown before⁴, an appreciable temperature decrease (of the order of several degrees) was calculated in the stratosphere while a slight but uniform temperature increase was calculated throughout the troposphere. The largest change in magnitude was $\sim -7.5^\circ\text{C}$ at 40 km, for a 33% ozone depletion (1996). This is similar to the results of McElroy *et al.*⁷ for the stratospheric temperature effects of

Fig. 1 Percentage of ozone decrease⁶, based on R11 and R12 increasing at a rate of 10% yr⁻¹ (model D).



SST fleet operations, who found a maximum temperature decrease of $\sim -10^\circ\text{C}$ at 40 km. In both cases the stratospheric cooling results from a decrease in the absorption of visible and infrared radiation by ozone.

The increased uncertainty in our calculated temperature change (Fig. 2) for 44 and 48 km reflects the difficulty in treating adequately the increasingly complex spectra of the optically active atmospheric constituents at high altitudes.

Our calculated tropospheric temperature changes were 0.04°C for 1979, 0.07°C for 1984 and 0.16°C for 1996, a roughly linear increase with time, at a rate of $\sim 0.06^\circ\text{C}/10\text{ yr}$. This slight surface heating is the result of more solar radiation being absorbed by the particulate layer which, in turn, heats and radiates more infrared radiation downwards to raise the surface temperature.

Similar calculations for the ozone profiles of ref. 8 give a surface temperature increase of 0.14°C for his case I and 0.15°C for his case II. In case I, Crutzen assumed a fixed yearly Freon release rate (equal to the world 1972 production rate) into the troposphere and determined that a steady state would be reached by the year AD 2055. Halocarbons would then be diffusing out of the troposphere into the stratosphere at the same rate at which it is being released

These calculations suggest that a drop of several degrees in the stratospheric temperature might occur by the end of the century if ozone depletion proceeds according to model D of Wofsy *et al.* More recent reaction rate constants are felt to reduce these ozone depletion rates by a factor of ~ 2 (see ref. 9) and it has been found¹⁰ that the photocross-sections for R11 and R12 are reduced at lower temperatures. In addition, it has been suggested that ClONO_2 may be of significance in the photochemistry of stratospheric O_3 . These effects, of course, were not included in the Wofsy's calculated ozone reductions, which I have used. The importance of this temperature effect on climate (if any) has not yet been established. Since previous calculations have indicated that the thermal effect may be strongly influenced by the altitude of the maximum in the ozone profile⁴, the results obtained here would be expected to vary with both latitude and season.

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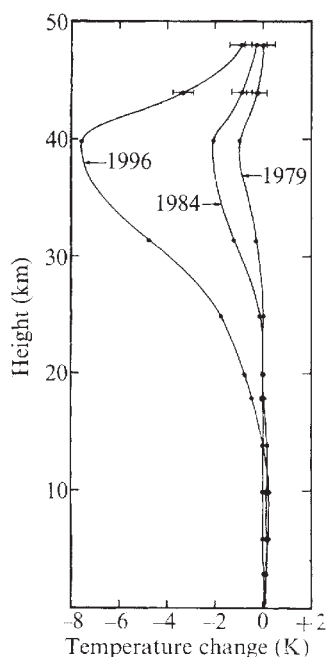


Fig. 2 Calculated temperature changes, based on the calculated ozone profiles of Wofsy, McElroy and Sze³. The 1972 temperature profile was calculated using the CO_2 and H_2O profiles described previously⁴, three layers of water clouds, a surface albedo of 0.097, a background low-lying particulate layer of optical density 0.065, and 4% Rayleigh scattering. The zenith angle and length of daylight were characteristic of 30°N latitude, July. Although the M-W model does not include general circulation, it does introduce a convective contribution when the lapse rate exceeds the thermodynamic adiabatic limit.

at the surface. In these steady-state conditions he calculated that the ozone would be reduced by 7.0% of its 1972 level. For production increasing at $10\% \text{ yr}^{-1}$ (Wofsy, model D) this change would be reached by 1987.

The Crutzen ozone profiles were also used to test the sensitivity of the temperature calculation to surface albedo. As the surface albedo was increased from 0.1 to 0.6, the stratospheric cooling increased $\sim 0.4^\circ\text{C}$ while the tropospheric heating decreased $\sim 0.06^\circ\text{C}$. Thus the dependence on surface albedo seems to be of relatively minor importance in the stratosphere and of greater importance for surface temperatures.

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A possible Himalayan microcontinent

ONE of the most significant features of the Himalayan mountain chain is its crystalline core which separates the tectonic belts of the Palaeo-Mesozoic-Cainozoic rock sequences of the Himalaya in the south and those of the Trans-Himalaya in the north. This crystalline core is described as the Central Crystalline Zone¹ and the Central Barrier². There are indications that the rocks of this zone are essentially a Precambrian-Proterozoic rock suite^{3,4}, although they were strongly remobilised during the Himalayan orogeny. No hypothesis on the evolution of this tectogene has yet explained satisfactorily the existence of the crystalline core^{4,5}, and taking this and the tectono-stratigraphic evidence into consideration and identifying some constraints in the existing models on the origin of the Himalaya, an alternative model, based on plate tectonics, involving microcontinents, is suggested here.

In the geosynclinal models the Central Crystalline Zone has been given distinct geotectonic status as the Central Himalayan geanticline⁶, intrageanticline⁷, and miogeanticline⁸, but its role in such models is uncertain. There have been attempts also to fit the stratigraphic and tectonic picture of the Himalaya into an Alpine framework^{9,10}. Such an attempt runs into difficulties because, although the Himalaya is a Cainozoic mountain chain, the extent and type of Mesozoic-Cainozoic sedimentary and tectonic record do not permit a satisfactory application of the classic Alpine geosynclinal model in the Himalaya¹¹. The bulk of the rocks in the Himalaya *sensu stricto* are reworked Precambrian/Proterozoic basement rocks, and Cainozoic granite-granodiorite massifs, which occur in the Axial and Inner Tectonic Belts¹², roughly defining the core of this chain (Fig. 1). A narrow zone of tectonised Palaeo-Mesozoic rocks, having a marked lateral inconsistency and strong stratigraphic breaks at places, occur to the south of these belts as allochthonous masses, thrust over the Cainozoic sediments of the Foothills. The Palaeo-Mesozoic sedimentary record is, however, fairly complete in the Tibetan zone of the

Trans-Himalaya. The most prominent and continuous dislocation zones are the Main Boundary Fault (tectonic contact of the allochthonous Pre-Tertiary rocks with the Tertiary rocks), Main Central Thrust (tectonic contact of the Axial gneisses and granites with the reconstituted Precambrian-Proterozoic rocks of the Inner Belt), Trans-Axial Thrust (tectonic contact of the Tethyan sedimentary sequence with the Axial rocks). North of the main Himalaya, the Indus-Tsangpo suture^{1,13} possibly defines an ophiolite-mélange zone. These dislocation zones and the character of the rock suites involved between them, seem incongruous in the regional perspective of eu-miogeosynclinal couple which is one of the fundamental tenets of the geosynclinal hypothesis.

In the plate tectonic model, the Himalaya is considered to be the classic example of a continent-continent collision system^{14,15} although such a system is not yet fully understood¹⁶. The application of such a model¹⁷ is faced with no fewer difficulties than those in the geosynclinal models. The plate tectonic models postulate the existence of a Tethys ocean, thousands of kilometres wide, separating India and Eurasia, before the foundering of the Gondwanaland^{13,18,19}, and therefore, the Himalaya is considered to have originated at the site of this consumed ocean. The crystalline axis of the Himalaya and the Palaeo-Mesozoic shallow marine and even continental sedimentary sequence to the South and North of this axis impart the main geological constraint to such models. Moreover, Tibet is mildly seismic²⁰ and no Tertiary to Sub-Recent volcanics are recorded in this region. The gravity data in the Himalaya clearly isolate it geophysically from the Trans-Himalayan region²¹. These facts do not fit well in a scheme involving direct collision between the Indian and Tibetan shields. Plate tectonic models are in a state of confusion, which can be realised from the fact that it is still a matter of conjecture and personal conviction as to where the junction between the Indian and Eurasian plates actually lies. The suggestions are that it lies along the Indus-Tsangpo suture¹³, the Main Central Thrust²² and even the Tien Shan²³. These difficulties have apparently led to the suggestion²⁴ of a Greater India in the Gondwanaland, and to the proposal²⁵ that the Himalaya might represent an intra-continental mountain chain. Because of this confusion, and

considering the stratigraphic, palaeontological and palaeomagnetic data, the idea of wide oceanic separation between India and Tibet has also been criticised^{25,26}.

It seems that many of these inconsistencies and difficulties in the concept of evolution of the Himalaya can be overcome if this tectogene is considered to have originated through the interaction of the Indian shield and a cluster of microcontinents, instead of a direct collision between the Indian and Tibetan shields, across the Tethys. Such an interaction would account for, among other geological and geophysical attributes which characterise the Himalaya and isolate it from other segments of the Mediterranean mobile belt, the crystalline core with Cainozoic granite-granodiorite intrusives, shallow marine and platform sediments on either side of this core. The presence of a microcontinent would also imply a reduction of effective width of the Tethys which is necessary^{23,24} to explain the geological picture of this region.

A strong tectonometamorphic break²⁷ in the Himalayan stratigraphy is indicated by extensive Permo-Carboniferous conglomerate and boulder-bed (Blaini-Rangit-Lachi). This break is linked with the Hercynian hiatus after which Gondwana sedimentation had commenced in Peninsular India. A correlation between the Precambrian-Proterozoic and Palaeozoic rocks in the Himalaya and those in the Indian Peninsula, and relict Peninsular structural grains in the Himalayan rocks^{1,28} point to an inheritance of the Indian shield elements by the Himalaya. The suggestion that the Himalaya is essentially a Hercynide fold belt²⁹ is also relevant in this connection. It is likely that Hercynian distension caused either opening or widening of the embryonic Tethys and that during this process rifting of the northern edge of India took place, so that a cluster of microcontinents came into existence between the main continents of India and Tibet (Fig. 2). This distension was responsible for the Gondwana graben system in India and Upper Palaeozoic sedimentary-volcanic sequence in Tibet. As this process continued, the oceanic separation between the microcontinent and the Tibetan shield increased to give rise to what may be designated as the Mesozoic Tethys. In the initial stages, the Himalayan basin³⁰ was a rift system and an embryonic ocean, which as indicated by Upper Palaeozoic basic

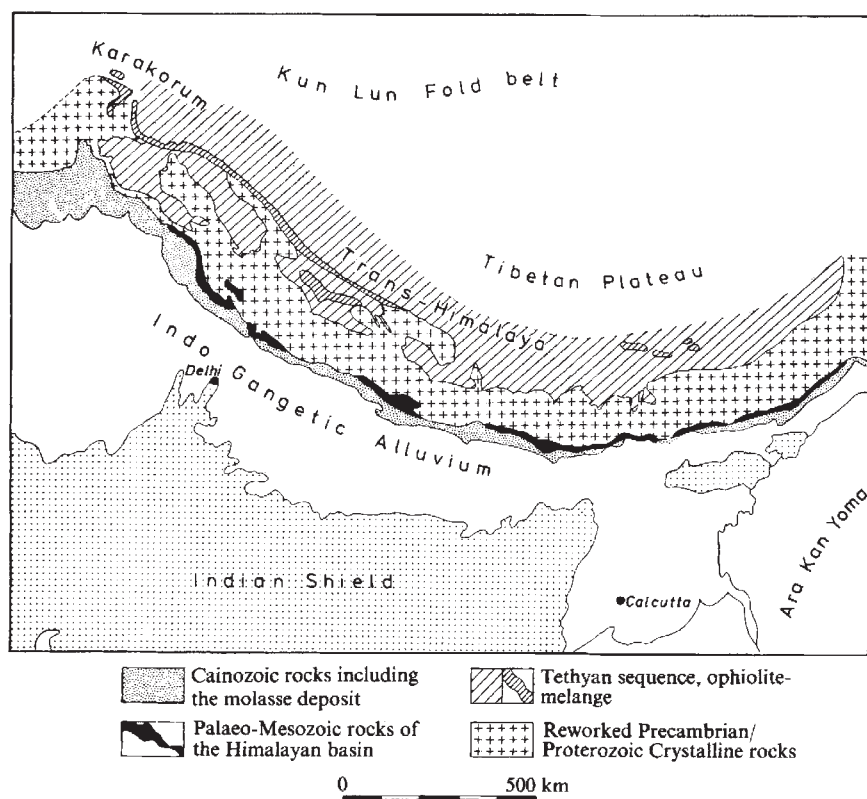


Fig. 1 Simplified geological map of the Himalaya (after ref. 1). Note extent of crystalline rocks in the core.

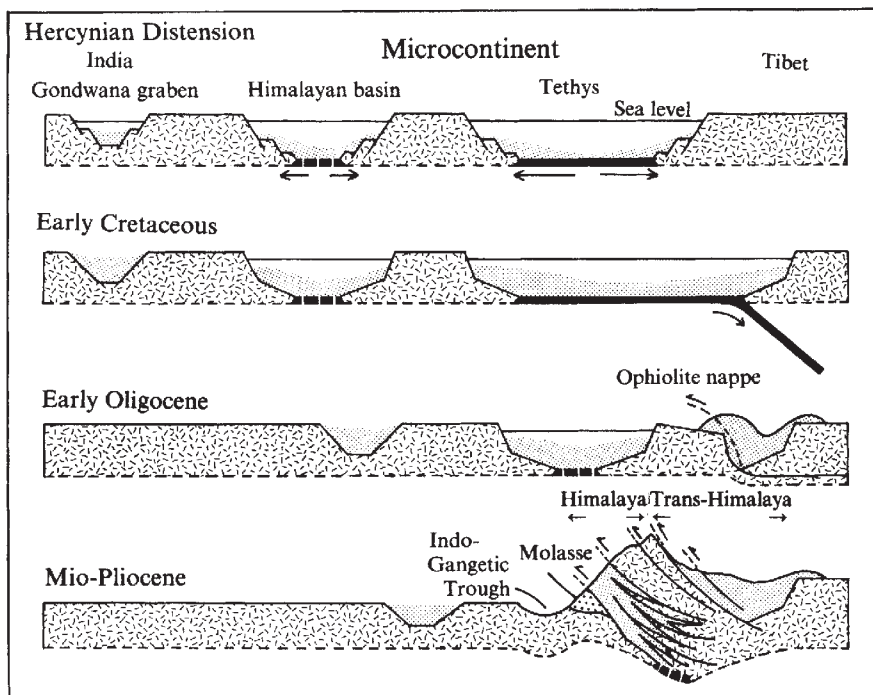


Fig. 2 Plate tectonic cartoon showing the evolution of the Himalaya with the involvement of microcontinent.

volcanics in Kashmir and Eastern Himalaya, contained mantle reaching fractures²³. Major and trace element geochemistry of these volcanics from a part of the Eastern Himalaya (unpublished data of the author) suggests that they are transitional between ocean floor and 'within plate'³¹ basic rock suite. Most probably, continued rifting and distension caused appreciable thinning of the continental crust and at a late stage, formation of an intermediate or oceanic crust in this basin. Rift tectonics are amply demonstrated by lateral inconsistency and temporal heterogeneity in Upper Palaeozoic and Mesozoic stratigraphy which ranges from marine, platform to even continental types. Local intermingling of Gondwanic fauna with the Eurasian types in the sediments of the Himalayan basin would suggest that the microcontinent did not behave as a solid barrier between the Tethys proper and the Himalayan basin. There might have been a few isthmuses.

In late Mesozoic time the Tethys began to shrink in consequence of northward drift of the Indian plate which carried along with it the microcontinent. In early Cainozoic period the northern edge of the microcontinent collided with the Tibetan shield along the Indus-Tsangpo suture. The Tethyan sediments were squeezed up and ophiolite-melange exotic blocks¹ were thrust to the south which now occur in association with the shallow marine sediments deposited at the northern edge of the microcontinent. Either a part of this microcontinent or similar continental blocks in the Tethys might have been thrust below Tibet to cause thickening of the continental crust in the latter. Because the orogeny had a south-directed polarity³², later in the collisional sequence the northern edge of the Indian shield collided with the microcontinent, probably after the suturing of the Tibetan shield and the microcontinent. As a result, the microcontinental basement rocks, reworked during this orogeny, were thrust over the folded and dislocated cover sediments of the Himalayan basin to the south. As indicated by the positive gravity anomaly, the oceanic slab of this basin probably lies slung beneath the mountain chain. It also seems likely that underthrusting of this slab may be linked with the generation of Cainozoic granite-granodiorite intrusive rocks in the Axial zone of the Himalaya, which are difficult to explain in plate tectonic models where direct collision between India and Tibet is postulated. The orogenic polarity is exhibited by the Plio-Pleistocene Siwalik molasse deposits which are contained in the linear trough at the down-buckled northern edge of the Indian shield, and which are deformed and overridden

by the allochthonous Palaeo-Mesozoic rocks, and in some cases, even by the Precambrian-Proterozoic rocks. The bi-phased collision scheme suggested here is supported by palaeomagnetic evidence³³ provided by DSDP cores which indicates a faster rate of northward movement of India from the Cretaceous to the Middle Eocene (the phase of the disappearance of Tethys and the collision of Tibet with the microcontinent), and a subsequent slower rate from early Oligocene (the phase of interaction between the Indian shield and the microcontinent). The Indus-Tsangpo suture and the Trans-Axial thrust have been virtually inactive in recent times, but the northerly thickened sediments in the Indo-Gangetic trough³⁴, the neotectonic features and the seismic pattern^{20,35} in the Himalaya suggest that the main movements are localised in the microcontinental domain, along the Main Central Thrust, and in the fold-thrust belt to the south.

It therefore seems appropriate to conclude that the Himalaya might indeed represent reconstituted and 'digested' upthrust microcontinental blocks which tectonically rest over the deformed cover rocks, deposited in the Himalayan basin. The Tethys, if it ever existed as a very wide ocean, as normally postulated, had little direct role in controlling the stratigraphic composition of this tectogene.

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The sea level in the last interglacial

$^{230}\text{Th}/^{234}\text{U}$ dating of coral terraces at 2–9 m above sea level on 'stable' oceanic islands has suggested that the sea level during the last interglacial, 120,000–140,000 yr BP (refs 1–3) was slightly higher than at present. However, accurate dating of interglacial shoreline facies on continental coasts far removed from plate boundaries has been largely neglected (the west coast of Australia¹ and Florida⁴ are exceptions). Attempts have been made to date interglacial shoreline deposits from SE Australia using molluscs⁵, but this has proved unreliable⁶. The recent discovery of buried corals at two localities on the E coast of Australia has provided an opportunity to determine more precise ages by $^{230}\text{Th}/^{234}\text{U}$ dating.

Multiple bay barriers composed of siliceous sand are a common geomorphic feature along the central and northern coasts of New South Wales. Two distinct barrier systems, the Inner Barrier and the Outer Barrier, have been recognised⁷. The Outer Barrier system is a Holocene feature, that has developed since sea level reached its present position ~ 6,000 yr ago⁸. The Inner Barrier system extends intermittently for almost 1,000 km along the coast north of Newcastle. Marine facies of this system occur at elevations of 2–8 m above present mean sea level, as determined by seams of heavy mineral and primary sedimentary structures formed on high energy, open coast beaches (tidal range ~ 2 m at spring tides). Lagoon facies are locally well developed and achieve maximum elevations of 4–6 m above mean sea level. There is no evidence of coastwise tilting of either facies in New South Wales.

The corals dated for this study come from two localities in association with the Inner Barrier, one near Newcastle (latitude 33°45'S; longitude 151°133'E), and the other near Evans Head (28°04'S; 153°27'E). In both cases the corals are in close proximity to bedrock promontories. Two coral species

from Newcastle were collected from an excavation 7 m below MSL at the base of a marine transgressive sand unit within the Inner Barrier. Corals from Evans Head were collected from an exposure behind the Inner Barrier on the south bank of the Evans River within present tidal limits. One specimen, identified as *Pocillopora damicornis*, was in its growth position, while others (*Acropora* sp., *Favites* sp.) seem to have been only slightly reworked. The corals and associated molluscan fauna suggest deposition in a shallow quiet-water lagoon, open to the sea.

The radiochemical data and ages of the corals are shown in Table 1. The corals from beneath the Inner Barrier near Newcastle give consistent ages of 143,000 and 142,000 yr BP ($\pm 12,000$). The corals from behind the Inner Barrier of Evans Head gives ages ranging from 112,000–127,000 yr BP; the *in situ* coral has an age of 118,000 $\pm 9,000$ yr BP. These dates confirm that the Inner Barrier was formed during the last interglacial. Of the five samples from Evans Head two are considered to be unreliable even though their ages agree reasonably well with the others. One coral (74630127) has a high uranium concentration and a high $^{232}\text{Th}/^{230}\text{U}$ activity ratio. The other coral (74630128) has a $^{234}\text{U}/^{238}\text{U}$ activity ratio greater than that of modern corals, and it also has a very high $^{232}\text{Th}/^{230}\text{Th}$ activity ratio.

The corals from Evans Head are ~ 20,000 yr younger than those from Newcastle. Evidence from New Guinea^{9,10}, Jamaica¹¹, and Timor (J. Chappell, personal communication), suggests that there were two stands of high sea level during the last interglacial at ~ 120,000 and 135,000 yr BP, with possibly a minor regression in between. The stratigraphy and morphology at the two coral sites in New South Wales indicate that the coral deposits near Newcastle could have been formed during a marine transgression before the 135,000-yr BP high sea level, while the Evans Head corals did not begin to grow until the second high sea level between 125,000 and 118,000 yr BP. Although the pattern of dates from Newcastle and Evans Head are generally consistent with those established from coral terraces of tectonically uplifted areas, the precise relations between dated corals, highest sea levels reached during the last interglacial, and deposition of the Inner Barrier, are not well enough understood to establish an interglacial sea level curve for eastern Australia. The relationships between barrier and lagoon facies are, however, clear enough to point to a sea level during the final phase of the last interglacial ~ 4–6 m above present sea level.

It is difficult to determine the precise elevation of interglacial sea levels on oceanic islands. The last interglacial terrace on oceanic islands varies between 2 and 9 m above present sea level¹. The unstable nature of many oceanic islands, and the uncertain position of dated corals with respect to sea level at the time of growth probably accounts for such differences. There is also the problem of the relationship between relative sea levels inferred from oceanic islands and those from continental coasts resulting from differences in their respective hydro-

Table 1 Radiochemistry and ages of corals from the Inner Barrier of New South Wales

Sample number	Coral species	Total U (p.p.m.)	$\frac{^{234}\text{U}}{^{238}\text{U}}$	$\frac{^{230}\text{Th}}{^{234}\text{U}}$	$\frac{^{232}\text{Th}}{^{230}\text{Th}}$	Age (10^3 yr)
Newcastle Bight						
74630115A	<i>Goniopora lobata</i>	2.73 ± 0.05	1.11 ± 0.02	0.75 ± 0.03	0.01	143 ± 12
74630115B	<i>Blastomussa wellsi</i>	3.30 ± 0.04	1.10 ± 0.01	0.74 ± 0.03	0.02	142 ± 12
Evans Head						
74630127	<i>Montipora</i> sp.	4.37 ± 0.07	1.13 ± 0.01	0.65 ± 0.03	0.05	112 ± 9
74630128	<i>Platygyra lamellina</i>	3.12 ± 0.08	1.18 ± 0.03	0.69 ± 0.07	0.28	122 ± 23
74630129	<i>Acropora</i> sp.	3.75 ± 0.05	1.11 ± 0.01	0.66 ± 0.03	0.02	114 ± 9
74630130	<i>Acropora</i> sp.	3.62 ± 0.07	1.13 ± 0.01	0.70 ± 0.05	0.02	127 ± 18
74630137	<i>Pocillopora damicornis</i> *	3.44 ± 0.05	1.11 ± 0.01	0.67 ± 0.03	0.02	118 ± 9

All samples are 100% aragonite.

* *in situ*.

isostatic behaviour^{12,13}. Nevertheless, for oceanic islands and continental coasts not located near plate margins the degree of difference may be $< \pm 2$ m. The maximum relative mean sea level for the unwarped New South Wales interglacial deposits based on elevations of lagoonal-tidal flat surfaces is 5 ± 1 m. This compares quite well with the recent estimate of 7.6 m for the 120,000-yr-old Waimanalo shoreline on Oahu, Hawaii³.

In New South Wales, there is no clear evidence for marine deposition older and higher than those of Inner Barrier age. The outer continental margin is subsiding at the rate of 0.01 m per 103 yr determined from seismic and bore data from eastern Australia, a figure comparable with similar continental margins elsewhere¹⁴. Therefore, it is likely that sea level returned approximately to the same position, or slightly lower, during each succeeding interglacial over at least the past 700,000 yr (ref. 15). In the documented spectrum of Quaternary sea level oscillations, the last interglacial appears to have been an exceptional event in so far as it was characterised by a great ocean water volume and warm sea temperatures^{15,16}. Inner Barrier elevation and faunal composition further support this conclusion that global ice volumes were smaller in the last interglacial than at present.

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Stab initiation of explosions

MECHANICAL initiation of fast reactions in solids can be achieved by various methods: impact^{1,2}, friction³, and shock⁴. Although it is now generally accepted that the initiation starts with the degradation of mechanical energy to heat, the precise mechanisms by which the energy conversion takes place are not understood clearly. I am concerned here with the initiation mechanism in a column of a compacted solid primary explosive when stabbed with a hard small angle metallic conical striker, which I show to be frictional. A model is presented, which, for the first time, gives a quantitative estimate of the maximum temperature rise generated during the impact.

The model is based on experimental investigations of the deformation behaviour of compacts of various reactive materials (silver azide, lead azide, lead azotetrazole) when hit with steel conical indenters. These indenters had semi-apex angle 15° , were polished to a surface roughness of $5 \mu\text{m}$ and had a flat tip of diameter $15 \mu\text{m}$. The compact of a selected material (particle size up to $25 \mu\text{m}$) was made in an aluminium cylindrical container, 4.3 mm in diameter, 4.8 mm high and 0.25 mm wall thickness, by placing a quantity of the material in the container and compressing with a tightly fitting cylindrical punch to a load

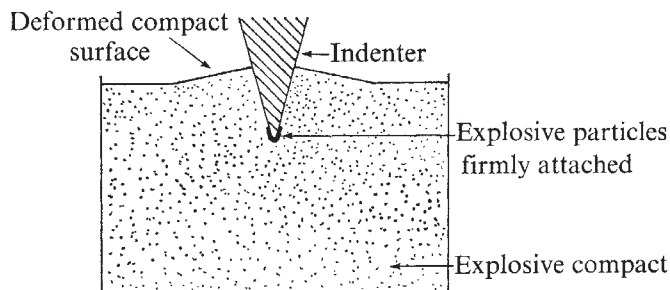


Fig. 1 Adhesion of particles to the indenter tip during penetration.

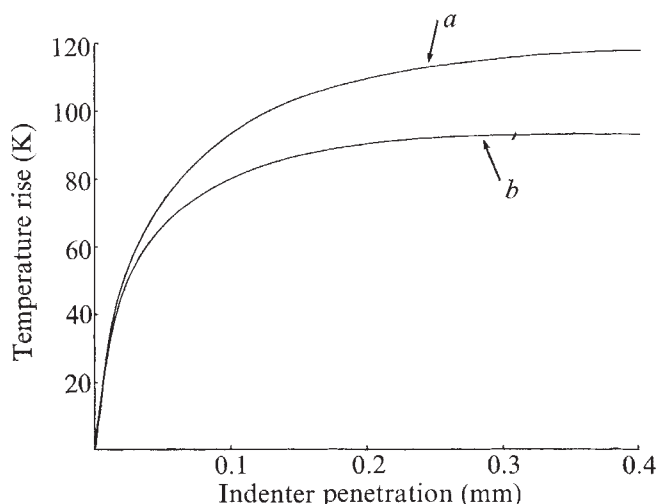
of 215 kg. Indentations were carried out using an Instron machine (crosshead speed 0.166 mm s^{-1}) and it was found that the load, L , and indenter penetration, x , were connected by

$$L = k_1 x^2 \quad (1)$$

where k_1 is a constant. An important observation was that during the indentation surface particles of the compact became firmly attached to the indenter tip and formed a thin coating on it (see Fig. 1).

Other studies on the deformation of compressed powders of inert materials with steel wedges (semi-apex angle 15°) revealed that the particle flow trajectory met the wedge surface at $\pi/2$, and this, combined with the observations described above (equation (1)), suggests that the deformation of the compact by small angle indenters can be described by plasticity theory⁵ with a high value of the coefficient of friction between the indenter and the compact. This means that during the whole penetration period, the particles firmly attached to the indenter surface will rub against bulk particles with a relative velocity of $v(1 - \sin^2 15^\circ)$, where v is the indenter velocity. On the other hand, the relative movement between the particles in other regions (that is, away from the indenter surface) of the compact will not occur for the whole period of the indentation. Moreover, as the thermal conductivity of the particles is much smaller than that of the indenter, the temperature generated at the metal/particle interface will be much smaller than at the particle/particle interface. Therefore, during indentation the maximum temperature rise will occur at the surface of the particles attached to the indenter.

Fig. 2 Calculated temperature rise against indenter penetration graphs when two conical indenters of different masses but having the same initial kinetic energy impact a column of compacted lead azide. *a*, Indenter mass 0.145 kg; velocity 0.5 m s^{-1} ; *b*, indenter mass 0.290 kg; velocity 0.353 m s^{-1} . Both indenters come to rest at a penetration of 1.16 mm. Note that the steady-state temperatures are reached well before the indenters come to rest.



To calculate the magnitude of the maximum temperature rise it is assumed that the particles attached to the indenter tip rub against a 'continuous plane surface' provided by the particles in the bulk. Now consider the case of one of the particles attached to the indenter. The frictional heat produced per second per unit area is

$$\frac{\mu v(1 - \sin^2 15^\circ) w_p}{A}$$

where μ is the coefficient of friction, A the area of contact, and w_p the normal force on the particle. For a compact, the normal force w_p for a particle of a given size can be calculated by assuming that the normal force on the surface of the indentation is uniform. The temperature rise can then be calculated by using the method of Blok⁶ (any temperature rise from chemical reactions is neglected). If the contact area is circular of radius R , and q_1 and q_2 are the fractions of heat going into the particle and the plane surface, respectively, (that is, $q_1 + q_2 = 1$), the temperature rise ΔT at the centre of the contact area after a time t is given by

$$\Delta T = \frac{4}{\pi^{\frac{1}{2}} \rho c} \int_0^t \frac{q_1 \mu v (1 - \sin^2 15^\circ) w_p (1 - \exp(-R^2 \rho c / 4t))}{2A(4\lambda t / \rho c)^{\frac{1}{2}}} dt \quad (2)$$

where ρ is the density, λ the thermal conductivity, c the specific heat and

$$q_1 = \left[1 + \sqrt{\left(\frac{\pi}{2} \frac{v(1 - \sin^2 15^\circ) R \rho c}{4\lambda} \right)^{\frac{1}{2}}} \right]^{-1}$$

Now we can calculate the maximum temperature rise when a conical striker (semi-apex angle 15°) of mass M is dropped on to a compact for which the value of the constant k_1 (equation (1)) has already been determined by experiment. The motion of the indenter will be given by

$$M \frac{d^2 x}{dt^2} = -k_1 x^2 \quad (3)$$

This can be integrated to give the indenter velocity as a function of time. Then, by using equation (2), we can calculate the temperature rise at any time (or penetration) during the impact.

As an example, the temperature rise is calculated when a conical striker, mass 0.145 kg, strikes a compact of lead azide (compaction pressure 14.8 kg mm^{-2}) with an initial velocity of 0.5 m s^{-1} . Lead azide has been chosen since all the parameters in equation (2) are known and are:

$$\begin{aligned} w_p &= 2.27 \times 10^{-3} \text{ kg (for a particle } 25 \text{ } \mu\text{m across)} \\ k_1 &= 3.53 \text{ kg mm}^{-2} \\ c &= 4.85 \times 10^3 \text{ J kg}^{-1} \text{ K}^{-1} \\ \lambda &= 16 \times 10^{-2} \text{ J m}^{-1} \text{ s}^{-1} \text{ K}^{-1} \\ \rho &= 4.7 \times 10^3 \text{ kg m}^{-3} \\ \mu &= 0.28 \text{ (for the case of plastically deformed contact, see ref. 7)} \end{aligned}$$

$R = 6.4 \text{ } \mu\text{m}$ (the value of R was obtained separately by compressing a lead azide particle against a rigid surface by a load equal to the normal force on a particle of that size during the indentation of the compact).

The results of the calculations are shown in Fig. 2. Curve *a* is for a 0.145-kg indenter, whereas curve *b* is that for a 0.290-kg indenter, but with the same initial kinetic energy as before. It will be seen from Fig. 2 that the temperature rise first increases quite rapidly with penetration, but its increase then slows down, and reaches a 'steady-state' value well before the indenter comes to rest. Note that the value of the steady-state temperature rise for the 0.145-kg indenter is 118 K, whereas it is 93 K for the 0.290-kg indenter and also that, for a given penetration, the 0.145-kg indenter gives a higher temperature. This explains the observations from stab sensitivity tests of an explosive carried out with indenters of different masses that the efficiency of

initiation of reaction is higher for the indenter of smaller mass.

The steady-state temperature for the 0.145-kg indenter is 118 K plus the initial temperature of lead azide (293 K), giving 391 K; the ignition temperature of lead azide is $\sim 620 \text{ K}$ and therefore initiation of reaction is not to be expected, as is found experimentally. The same impact conditions are, however, just sufficient to cause initiation of reaction in lead azotetrazole whose ignition temperature is 493 K. The coefficient of friction for lead azotetrazole is twice that for lead azide, but the other physical properties of the two materials are similar. The estimated steady-state temperature is $\sim 530 \text{ K}$. Therefore we expect a higher ignition probability for lead azotetrazole than for lead azide.

Our model also predicts that reaction initiation should occur at the indenter tip after the indenter has penetrated a certain minimum distance (for a given initial velocity of the indenter). High speed photographic studies of initiation of reaction in compacts of lead azotetrazole support these predictions, and a detailed account will be published elsewhere.

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Novel electrochemical reactor

THE past decade has seen a revival of interest in most areas of electrochemical technology because of its relevance to some of the more important problems of modern society, such as environmental pollution, scarcity of natural resources and energy conservation. Electrochemical processes are quite unique in the sense that they do not need any chemical to carry out oxidation reduction reactions, instead the energy transfer is by an inert electrode. Consequently processes are generally 'clean' and do not create undesirable waste products.

The major limitation for the widespread use of electrochemical processes has been the unsatisfactory development of electrochemical reactors, but recently¹ there has been increased activity in this area. Most electrochemical processes are limited by mass transfer rates, and attempts have been made to develop reactors with greater mass transfer capabilities, for example the fluidised bed, the packed bed and other three-dimensional particulate reactors^{2,3}. The limitations of these are well documented^{4,5}, especially the non-uniformity of electrode potential generated by poor electronic contact between the particles.

We have developed a novel electrochemical reactor based on packed carbon fibres as the working electrode. The primary design characteristic in this reactor is the increase of mass transfer rates while simultaneously having a controllable and uniform electrode potential over the entire electrode surface. The carbon fibres, which are produced by the thermal decomposition of polyacrylonitrile, have very favourable properties as electrode materials because of their high electrical conductivity and hard vitreous surface. They also show favourable hydrogen and

oxygen overvoltage characteristics with a working range of -1.4 to $+1.2$ V SCE in neutral solution. This gives a wide window which is especially important in the anodic range. The most outstanding property of carbon fibres for our use is their enormous surface area. Fibres are typically $5\text{--}10\text{ }\mu\text{m}$ in diameter so that 1 g of carbon fibre has a surface area of $2.6 \times 10^6\text{ cm}^2$ (ref. 6). This is $\sim 1,000$ times larger surface per unit volume than other types of particulate reactor.

As a result of the increased surface area, the mass transfer rates in this type of reactor approach those achieved by some heterogeneous catalytic reactors; as a consequence it should have immediate impact on a wide range of electrochemical processes which were hitherto economically and technologically unfeasible. As an example we can cite an application of environmental importance, the electrowinning of trace toxic metals from effluent.

The reduction of Cu^{2+} was taken as an experimental reaction, and it was found that for a 100 p.p.m. solution at a flow rate of 3.0 ml min^{-1} a 99.3% conversion was achieved in a single pass through the reactor with a 12-s residence time. This compares to a residence time of 1–4 h for 95% conversion in packed bed and parallel plate reactors. This performance is several orders of magnitude greater than anything achieved by other reactor designs, the major improvements in the mass transfer rates arising from (1) the large surface area of fibres, (2) the low porosity and low adsorption characteristics of the fibres and (3) the fluid flow profiles in the reactor.

Scale up studies of the reactor are currently in progress, keeping the ratio of electrode area to reactor volume constant, and reactors of 10 l h^{-1} capacity are now functioning. The optimum bed depth of carbon fibres is 1 cm. Although the primary design consideration was for mass transfer controlled reactions, it has been found advantageous as well to carry out kinetically controlled reactions in this reactor. Various electrochemical processes are being investigated using this reactor and in all of them the major advantages are:

(1) The reactor can operate at lower current densities without corresponding decrease in yield, resulting in a sharp decrease in the cost per unit of production.

(2) It is possible to operate reactions with high yield at realistic time scales.

(3) The decrease in capital cost because they can be operated in a single pass process by having appropriate design parameters.

This should lead to substantial cost saving for all processes. Details of further work with this reactor will be reported in later publications.

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Equilibrium bicontinuous structure

BICONTINUOUS partitioning of a volume is produced by inscribing a continuous, orientable surface of positive genus without self-intersection. This divides the volume into two multiply connected, interpenetrating subvolumes, each of them physically continuous (mathematically connected). Neither on a line nor in the plane is there an analogue. A bicontinuous structure is a bicontinuous partitioning in which each subvolume is filled with a distinct, not necessarily uniform composition or state of matter. In familiar examples one subvolume is solid or semi-solid, for example sandstone or sponge. An interpenetration of two phases is bicontinuous only if each phase is connected across the specimen.

The idea put forward here is that bicontinuous structures may arise in fluids. The possibility of fluid analogues of porous media seems not to have been noticed before. The writer finds no mention of it in the literature on macroemulsions, periodic colloid structures, microemulsions, micellar solutions, mesomorphic phases and lyotropic crystals. Microemulsions, micellar solutions and mesomorphic phases are equilibrium states that typically consist of appreciable amounts of water, hydrocarbon and amphiphile^{1,2}. Some display cubic symmetry, which is interpreted in terms of discontinuous submicroscopic globs of one composition dispersed in a continuum of another³. An alternative is a periodic bicontinuous structure. For thermally disrupted, non-periodic bicontinuous structures, leading candidates are optically isotropic, light-scattering microemulsion phases containing comparable amounts of water and hydrocarbon^{1,4}; when one predominates, the other is often solubilised within discrete micellar structures of amphiphile. In microemulsion saturated with water and hydrocarbon, however, there is evidence that if discrete structures exist they are neither globular, tubular nor lamellar on the average and the continuous zone is dominated by neither water nor hydrocarbon⁵. The structural scale is beyond resolution by light microscopy and probably falls in the range 5–80 nm.

Intermolecular forces can conspire to elevate three energy effects to rule structure in this range. One is a sharp energy minimum when water and oil are everywhere segregated by a sheet of oriented amphiphilic molecules, the sheet constituting a partitioning surface, whether continuous or discontinuous. The second is a monotonic decrease of energy with decreasing area of the partitioning surface. The third is a sharp energy minimum with respect to the lattice parameter and symmetry of periodic structures, or mean scale length in partly randomised, isotropic structures. These three effects lead to a problem of constrained minimisation of area.

Fig. 1 Process for transforming a simple cubic array of spheres into a periodic bicontinuous structure of fused truncated octahedra with the same symmetry, lattice distance and volume fraction of one-half. The net area change is a decrease of 15%.

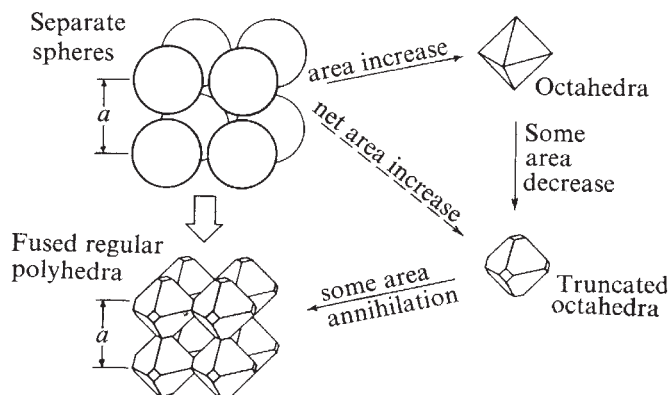


Table 1 Fused polyhedra compared with dispersed spheres in cubic arrays

	Simple cubic	Body centred	Face centred
Polyhedral subvolume	Truncated octahedra	Corner-notched	Truncated cuboctahedra
Complementary subvolume ('voids')	Truncated octahedra	Cubes and slabs	Truncated tetrahedra and truncated cubes
Volume fraction when areas are equal	0.40	0.53	0.57
Volume fraction when spheres are close-packed	0.52	0.68	0.74
Volume fraction when complementary subvolume becomes discontinuous	0.83	1	0.91
Area ratio when volume fraction is $\frac{1}{2}$	0.85	1.04	1.07
Range of volume fractions at which fused polyhedra have less total area	0.40–0.52 0.48–0.60	0.32–0.47 0.53–0.68	0.26–0.43 0.57–0.74

Calculations show that, for cubic symmetries, there are intermediate ranges of the ratio of the two subvolumes, within which a bicontinuous structure is favoured over dispersion of either subvolume as separate spheres. Examples are fused polyhedra with the same cubic symmetry and lattice parameter as the arrays of spheres, each polyhedron having originally as many vertices as the coordination in the array. Areas at fixed volume fractions are compared in Table 1; a process for making comparisons is pictured in Fig. 1. Notice that with body-centred and face-centred lattices there is a gap around volume fractions of $\frac{1}{2}$, in which dispersed spheres are again favoured.

Fused polyhedra represent upper bounds on partitioning area when symmetry, lattice parameter and volume ratio are fixed: the area can be further reduced by rounding edges and warping faces. The ultimate is a periodic minimal surface^{6,7}. According to an unreferenced note⁸, 17 intersection-free, infinite, periodic surfaces of cubic and other symmetries have been discovered. Portions of two are shown in Fig. 2 (refs 8, 9). With few exceptions⁷ areas and volume fractions are unreported. Nor has their stability been studied. 'Almost periodic' and otherwise less regular minimal surfaces of high genus seem not to have been examined mathematically.

Minimal surfaces have no mean curvature and satisfy the Young–Laplace equation for menisci between immiscible bulk phases at the same hydrostatic pressure. Thus minimal surfaces of high genus might occur, if only transiently, as part of the interface in a macroemulsion, particularly in the phase-inversion range.

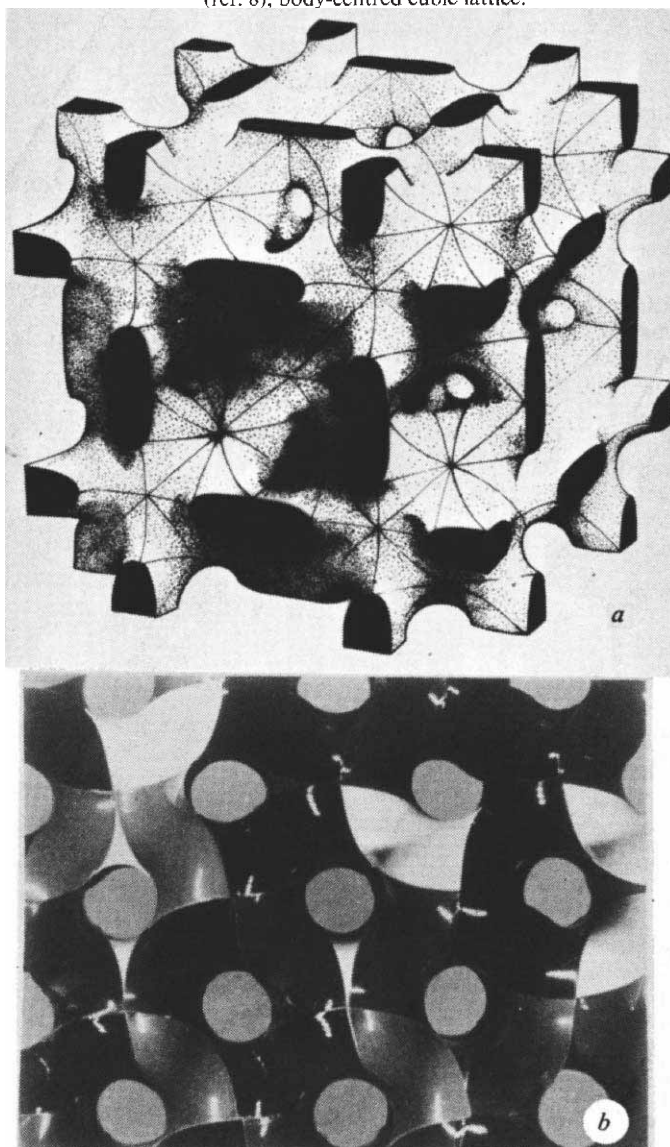
To account for variations in microemulsion properties Winsor¹ hypothesised that an amphiphile-dominated sheet, or partitioning surface, tends to become concave towards its hydrophilic or oleophilic side. In the concept of equilibrium bicontinuous structures this is equivalent to a fourth energy effect, a dependence of energy on curvature invariants (ref. 10, and M. L. Robbins, unpublished) or, in the extreme, a constant, non-zero mean curvature as a constraint. The minimisation problem becomes more difficult, and evidently has not been investigated even for periodic surfaces.

Equilibrium is governed by free energy, which encompasses entropic contributions besides energy effects. Thermal motions have to be accounted for in principle by identifying all energy states accessible to the system, in practice only those with energies close to that of the most probable state. Statistical thermodynamics indicates that thermal fluctuations can alter lattice distance and symmetry in periodic bicontinuous structures and that as temperature rises, periodicity and symmetry can be destroyed by the ever-greater shape excursions by the partitioning surface. On microemulsion scales these excursions are likely to cause connectivity fluctuations through pinching off and rewelding of narrow necks. As composition or temperature change and the energy minimum grows shallow compared to kT , this action can gradually yet completely destroy the continuity of one of the subvolumes, and then further reduce its mean coherence length until it is a dispersion of globs in the other subvolume. This may explain a striking feature of water–oil–amphiphile microemulsions: their continuous evolution into

a water-continuous solution of oil-swollen globular micelles on dilution with water, and into an oil-continuous solution of water-swollen inverted micelles on dilution with oil^{1,2}.

Bicontinuous structures may be present in mesomorphic, liquid-crystal-like phases regarded as dispersions of spheres, cylinders or lamellae. As with microemulsions, experiments are needed which discriminate unequivocally among the possibilities. Basic mathematical investigations of certain genus surfaces are indicated. Statistical physics can be brought to bear on what

Fig. 2 Examples of intersection-free, periodic, minimal surfaces, (a) Neovius's (ref. 9), simple cubic lattice; (b) Schoen's gyroid (ref. 8), body-centred cubic lattice.



may be a state of matter related to ordinary liquids as porous media are to homogeneous solids¹¹.

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Why be an hermaphrodite?

MANY animals and most higher plants are hermaphrodites. The basic argument of this paper is that the sex habit of a species is determined by selection acting on the number of offspring produced by individuals of different types. The argument differs radically from most earlier explanations of the evolution of hermaphroditism (reviewed by Ghiselin)^{1,2}, although it is formally similar to a recent explanation³ of sequential hermaphroditism, in which individuals function first as one sex and then the other.

Our fundamental assumptions are as follows. (1) Genes in a zygote can act as switches, directing development into one or other type (male, female, hermaphrodite), or, in hermaphrodites, can alter the relative allocation of resources to male and female functions. (The theory does not apply if sexual type is determined by cytoplasmic factors, as is sometimes the case for male sterility in plants.) (2) The total production of male plus female gametes by an individual is constrained to lie within a "fitness set", which cannot be altered by genetic change. It will be shown how the form of the fitness set determines the sex habit.

Consider, for concreteness, a plant species, and let m , f , and h be the numbers of male, female and hermaphrodite individuals

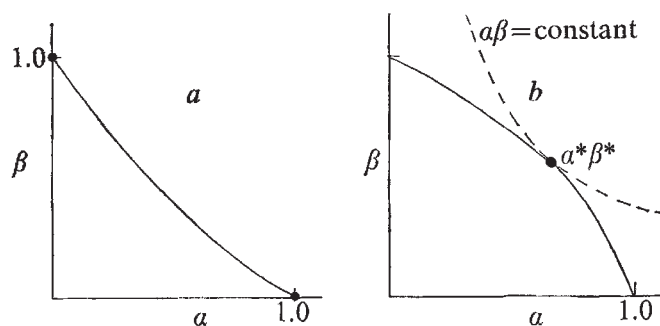


Fig. 1 *a*, A "fitness set" for the allocation of sex function. α is the pollen produced by an hermaphrodite, as a fraction of the total produced by a male individual. β is the corresponding parameter for a female. All possible values are assumed to be to the interior of the curve. With a concave curve, the hermaphrodite population is not an ESS, but a dioecious population is. *b*, Optimal resource allocation for an hermaphrodite. With a convex tradeoff curve, the hermaphrodite is an ESS. The ESS resource allocation ($\alpha^*\beta^*$) is the point on the curve which maximises the product $\alpha\beta$.

respectively in a population (counted at conception). For simplicity all individuals are assumed to have the same survival rate to adulthood. A male can produce N pollen grains, a female can produce n seeds, and an hermaphrodite αN pollen grains and βn seeds. The fitness set can then be represented as a graph of α against β , as in Figs 1 and 2.

Suppose the population produces R offspring. Assuming self-incompatibility, and using "fitness" to mean the expected number of offspring produced by an individual,

$$\text{fitness of a male} = R/(m + ah),$$

$$\text{fitness of a female} = R/(f + \beta h),$$

$$\text{fitness of a hermaphrodite} = R[\alpha/(m + ah) + \beta/(f + \beta h)].$$

If the situation is to be evolutionarily stable, two conditions must be satisfied: (1) the fitnesses of any types actually present in the population must be equal, and (2) the values of α and β in hermaphrodites, if present, must be an "evolutionarily stable strategy," or ESS⁴; that is, the actual phenotype, ($\alpha^*\beta^*$), of the hermaphrodites present in the population must be as fit as or fitter than any mutant phenotype ($\alpha\beta$) lying in the fitness set.

Case 1. $h = 0$. Population dioecious. By condition (1), $R/m = R/f$, or $m = f$. This is the familiar conclusion that the primary sex ratio is 1:1 when the costs of male and female offspring are equal⁵.

A dioecious population can be invaded by an hermaphrodite mutant if $\alpha/m + \beta/f > 1/m$; that is, if $\alpha + \beta > 1$. It follows that a dioecious population is stable only if the fitness set is concave (Fig. 1*a*). If it is convex (Fig. 1*b*), then only the hermaphrodite population is stable.

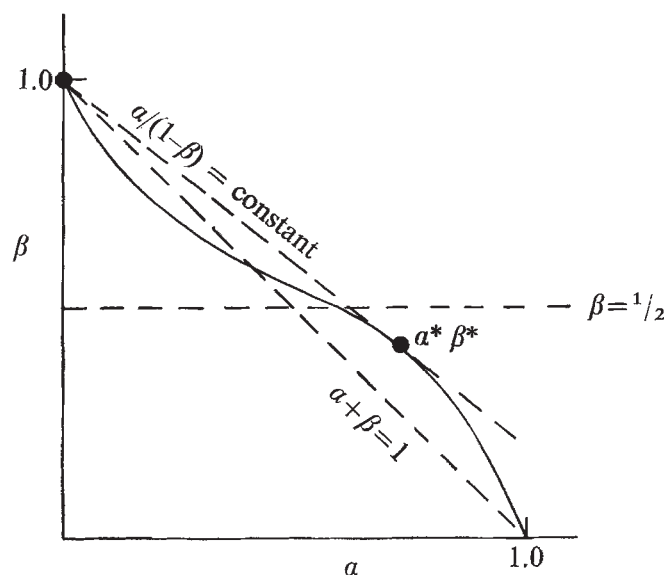


Fig. 2 An ESS which is a mixture of sexual types. If the curve is convex-concave, it may be possible for a pure sex (in this case a female) to invade. The resulting mixture is stable if (i) $\alpha^* + \beta^* > 1$, (ii) $\beta^* < 0.5$, (iii) $\alpha^*\beta^*$ is the point which maximises $\alpha/(1-\beta)$. This curve illustrates gynodioecy.

Case 2. $m = f = 0$, $h = 1$. Population hermaphroditic. If ($\alpha^*\beta^*$) is the phenotype of typical members of the population, and ($\alpha\beta$) of a rare mutant, then ($\alpha^*\beta^*$) is an ESS if the fitness of ($\alpha\beta$) is less than or equal to that of ($\alpha^*\beta^*$) for all points on the boundary of the fitness set. For stability against small perturbations, this requires that $(\alpha/\alpha^* + \beta/\beta^*)$ be at a local maximum when $\alpha = \alpha^*$, $\beta = \beta^*$. This is equivalent to the requirement that the product $\alpha^*\beta^*$ be a maximum (Figure 1*b*).

This is analogous to MacArthur's⁶ conclusion that selection will maximise the product of the number of males and females in a dioecious species. If the fitness set is bounded by the line $\alpha + \beta = 1$, as will be approximately true if pollen and seed production are limited by the same resources, then the ESS is for an hermaphrodite to divide its resources equally between pollen and seeds⁷.

Case 3. $h \neq 0$, $f \neq 0$, $m = 0$. Population gynodioecious. The criterion $\alpha\beta^*$ maximal provides a local stability criterion for a hermaphrodite population. But can a population with $h = 1$ be invaded by males or females? It can be invaded by females if $R/\beta^* > 2R$, or $\beta^* < 0.5$. Provided, however, that $\alpha + \beta > 1$ for some part of the fitness set, hermaphrodites will not be completely eliminated.

Figure 2 shows a fitness set for which the ESS is gynodioecy. Let $(\alpha\beta^*)$ be the stable phenotype of the hermaphrodites. Then the stable sex ratio, obtained by equating the fitness of females and hermaphrodites, is given by $f = h(1 - 2\beta^*)^8$. This implies an excess of hermaphrodites, as is in fact observed in natural populations⁹. It also implies that as the frequency of females increases, the resource allocation by the hermaphrodite shifts towards male function. This shift has also been observed¹⁰. The fitness of a rare mutant with a different resource allocation, $(\alpha\beta)$, is given by

$$V = R[\alpha/(m + \alpha^*h) + \beta/(f + \beta^*h)] \\ = R[\alpha/\alpha^* + \beta/(1 - \beta^*)]/h$$

The condition for $(\alpha\beta^*)$ to be an ESS is that V should be at a local maximum; which is equivalent to the requirement that $\alpha/(1 - \beta)$ should be at a maximum when $\alpha = \alpha^*$, $\beta = \beta^*$. The ESS can therefore be found by drawing a tangent as in Fig. 2.

The conditions for androdioecy, $h \neq 0$, $f = 0$, $m \neq 0$, are similar. All three types can coexist only in the artificial and unlikely case, $\alpha + \beta = 1$ exactly.

The conclusions can be summarised by saying that we would expect to find monoecy or hermaphroditism in species for which the fitness set is convex, dioecy if it is concave, and androdioecy or gynodioecy if it is concave-convex.

Are there any general reasons why fitness sets should be convex? We can offer three suggestions. (1) Low mobility: in animals, Ghiselin^{1,2} has noted that hermaphroditism tends to occur in species with low adult mobility. Low mobility will tend to be associated with a convex fitness set because in such species there will be little sexual dimorphism (except perhaps dwarf males) since males will not involve special locomotory or aggressive structures for seeking out and holding females. This means that a single individual can effectively serve both functions. Furthermore, low mobility limits male reproductive success—the number of eggs that a male can competitively fertilise (or an hermaphrodite acting as a male) should rise at less than a linear rate with resource input into male function. Note that this model assigns a different role to low mobility than the classical “low density model” as developed by Ghiselin^{1,2}. Even if all the eggs produced in the population are fertilised, the convex fitness set implies hermaphroditism.

(2) Low resource overlap: in plants pollen production occurs earlier in the season, for each species, than seed maturation. The two processes therefore depend, in part, on different resources, and one would therefore expect a hermaphrodite to do better than a linear combination of male and female. In animals, brooding will imply a convex set because female expenditure will occur later than male expenditure. Ghiselin¹ has pointed out that hermaphroditism in animals tends to occur in immobile species in which individuals brood the young.

(3) Cost sharing: in insect-pollinated plants, some energy must be expended by all types on producing organs of attraction which may serve both male (pollen) and female (ovule) function. If a plant's reproductive success tends more to be limited by its ability to attract pollinators than its direct input of resources into gametes, the fitness set will tend to be convex. The conclusion depends on assumptions, which merit further investigation, on how a plant allocates resources between organs of attraction and more directly reproductive structures. It does, however, suggest that dioecy should be rarer in insect-pollinated than in wind- and water-pollinated species.

We do not claim that the selective forces associated with resource allocation are the only ones relevant to the evolution of hermaphroditism. In plants, dioecy may evolve as a mechanism to prevent self-fertilisation¹¹. When associated with self-fertility, hermaphroditism may be an adaptation to situations (for example, parasitic and sessile animals; annual, monocarpic and colonising plants¹²) in which opportunities for cross-fertilisation are rare^{1,2}. We have also ignored possible effects on resource allocation of factors such as sperm storage (which is common in invertebrates^{1,2}) and pollen or sperm competition. Models considering these factors are now being developed and will be published elsewhere. The relative importance of these various selective forces can be determined only by comparative studies of the distribution of the sex habit, which we are undertaking.

The model outlined in this paper, and its biological interpretation, was developed independently in Utah and at Sussex.

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Tip formation is regulated by an inhibitory gradient in the *Dictyostelium discoideum* slug

THE cellular slime mould *Dictyostelium discoideum* (*Dd*) has proved suitable material for investigation of pattern formation. The morphogenetic field controlling *Dd* aggregation is now uniquely well characterised^{1–6}. Whether its characteristics are unique or universal is not known. Here, I help to put the slime mould into a general perspective by comparing its pattern formation with that of another well studied organism. I have used grafting experiments to investigate secondary axis regulation in the *Dd* slug. The experiments parallel the use of grafts by Wolpert and collaborators to elucidate hypostome regulation in hydra^{7,8}. They show that hydra and the slug use formally similar mechanisms for axis regulation. The known features of the *Dd* aggregation control system suggest that this does not account for axis regulation in the slug. (Details of the experimental procedure are given in Fig. 1 legend.)

A tipless slug (2345) was grafted with correct polarity to a range of anterior segments. This was to test the capacity of posterior cut surfaces at various levels in the slug to inhibit tip regeneration. The result (Fig. 2a) shows a posteriorly decreasing

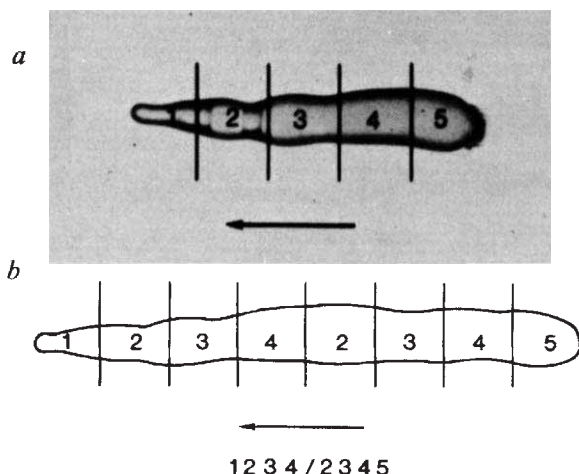


Fig. 1 Like hydra, the *Dd* slug has one obvious axis of polarity (its major axis), and a terminal organiser or dominant region (the anterior tip)^{9,10*}. A second tip inserted into a slug can organise a new axis. Tip excision delays development till a new tip is regenerated. If the excised tip is replaced, regeneration is usually inhibited. Axial grafts were used to investigate the inhibition process. *a*, Large slugs (1–3 mm) were taken from growth plates and laid on non-nutrient agar⁹NC4. They were measured into five sections of equal-length (1 anterior→5 posterior) and cut at one or more section boundaries, to make desired segments (for example, 1 and 2345). *b*, Grafts were made by joining the cut surfaces of a pair of slug segments as described in the text. Anterior and posterior graft segments were always from different members of a pair of slugs approximately matched for size. Most grafts were repeated 50–100 times. Chimaeric slugs resulting from grafts usually migrated for some time before making a fruiting body.

*Hydra is bipolar, with two terminal organisers (the distal hypostome and the proximal foot). The slug is probably unipolar. Here, I compare tip regulation in the slug with hypostome regulation in hydra.

gradient in capacity to inhibit a secondary axis (linear regression: first degree coefficient=0.22). A similar result is found for hydra^{7,8,11}. The data give a reasonable fit to a linear regression line ($P > 99.5\%$) but the fit is not significantly improved for higher degree polynomial plots. Two possible trivial explanations of this result were tested and eliminated (Fig. 2*b*, Table 1).

The results above suggest that *Dd* slugs contain an axial gradient in a parameter that inhibits tip formation. If this is true, we may also expect a gradient in threshold for a response to the inhibition. The low level of inhibition at region 4/5, which is demonstrably insufficient to inhibit tip formation by region 2 (high threshold), might thus inhibit tip formation by region 5 (low threshold) in the intact slug. The results of grafts to test this prediction are shown in Fig. 2*c*. They show a posteriorly decreasing gradient in threshold, exactly as in hydra^{7,8,11}. The gradient is probably nonlinear (improved fit to a second degree polynomial just significant at the 5% level).

In hydra, secondary axis inhibition is reduced if the host organiser (hypostome) is removed just before making an axial graft^{7,8}. Thus, a hydra H12/1234B56F graft makes two new hypostomes (one from each 1 region) but an H12/1234/B56F graft makes no new hypostomes and retains only the original hypostome. This result, and others, which parallel my findings in *Dd* have been interpreted (by Wolpert)⁸ in terms of the following concepts.

Hypostome inhibition is proposed to be caused by a fast regulating diffusion gradient of an inhibitory substance (I). I is secreted by the hypostome and destroyed elsewhere in the hydra. The I gradient interacts with a slowly regulating gradient of similar form in a cell state variable (P). P sets the threshold for a response to I. Interactions between I and P lead to hypostome formation in a predictable way. Removal of the host hypostome causes a fall in I and enhances secondary hypostome formation at local P maxima caused by grafting. It also causes a relatively faster fall in I at the anterior end of the hydra, where

I and P concentrations are initially high, with consequent formation of an anterior hypostome. The model thus provides a rule for polarity.

Tipless slug segments were compared with their intact (tipped) homologues as inhibitors of secondary axis formation. We also tested segments lacking 12 and even 123 to seek evidence for a distributed source of tip inhibitor (Table 2). We found that removal of the slug tip never reduces secondary

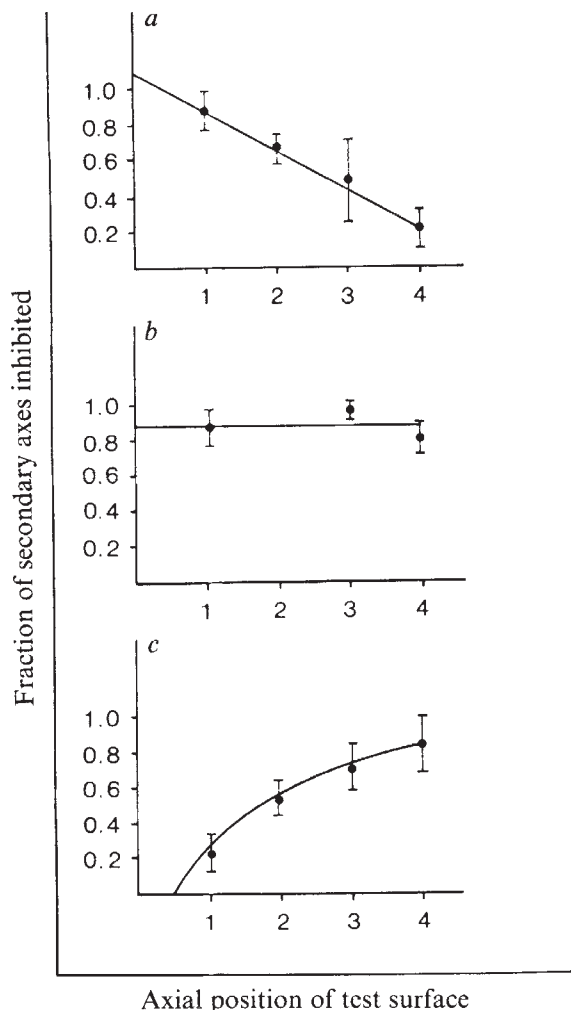


Fig. 2 Results of some grafting experiments. Ordinate, inhibition of secondary axes (axes formed by the posterior slug segment); abscissa, axial level of the cut surface in the test segment. 1, Cut between segments 1 and 2; 2, cut between segments 2 and 3 and so on. The data were obtained as results of individual experiments (8–15 grafts each). The frequency of inhibition obtained in each experiment was used as a data point to calculate regression lines, means and standard deviations. Linear regression plots are shown for 2*a* and 2*b*; a second degree polynomial for 2*c*. *a*, Test (anterior) segments 1,12,123,1234 were grafted to the standard posterior segment 2345. The plot shows a (posteriorly decreasing, approximately linear) dependence of secondary axis inhibition on the level of the cut surface in the test segment. This suggests a gradient of inhibition in the slug. Numbers of grafts for this experiment were: 1:82 2:82 3:68 4:78. *b*, A test of the possibility that the observed gradient in secondary axis inhibition reflects a gradient in adhesiveness between front cells and progressively posterior cells in the slug (with a consequent gradient in frequency of isolation of the posterior segment). The posterior segments 2345, 45, 5 were grafted to a standard anterior segment (1). These grafts show no monotonic dependence of secondary axis inhibition on level of the cut surface in the test segment (linear regression: first degree coefficient = 4.57×10^{-3}). The possibility was eliminated. Numbers of grafts for this experiment: 1:82 3:58 4:40. *c*, Test (posterior) segments 2345, 345, 45, 5 were grafted to the anterior segment 1234. The plot shows a posteriorly decreasing gradient in the capacity of the test surface to withstand inhibition by the anterior segment. This suggests a gradient in threshold for inhibition of secondary axes in the slug. Numbers of grafts for this experiment: 1:78 2:70 3:63 4:64.

axis inhibition and neither does removal of segments back to and including segment 3. Two explanations are possible within Wolpert's type of model. (1) The *Dd* I gradient shows considerable local homeostasis, or I diffuses very little. The tip and other distant regions are thus unimportant as a short term source of local I. P shows more homeostasis or diffuses less than I. (2) The tip is a localised source of fast diffusing I. As in hydra, organiser (tip) removal causes a general fall in I and enhances organiser formation at local P maxima. The parameters of organiser determination and differentiation (to inhibitory competence) differ, however, between hydra and the slug. Specifically, determination and differentiation are well separated in time in hydra and not in the slug. Two slug tips developing close together may thus compete (inhibit each other). In contrast, adjacent hydra hypostomes can usually develop independently.

The second hypothesis predicts that the tip of a single chimaeric slug developing from an appropriate graft (with a tipless anterior segment) may sometimes come from the posterior

Table 1 Effect of slug length on tip inhibition

Graft	Fraction of secondary axes inhibited	No. of grafts	Mean fraction of inhibition
1234/2345	0.25	8	0.21
	0.20	10	
	0.17	12	
	0.0	12	
	0.33	12	
	0.20	15	
1234/23	0.33	9	0.15
	0.25	8	
	0.13	8	
	0.0	10	
	0.0	8	
	0.38	8	
12/2345	0.10	10	0.74
	0.20	10	
	0.77	13	
	0.60	10	
	0.60	10	
	0.73	15	
	0.60	10	
	0.80	10	
	0.79	14	
	0.88	8	
	0.88	8	
	0.75	8	

To test the possibility that the observed gradient in secondary axis inhibition arises because artificially elongated slugs (with intercalary inserts) are mechanically unstable, the grafts 1234/2345, 1234/23 and 12/2345 were compared with each other. 1234/23 gave no more inhibition of secondary axes than 1234/2345 (greater length, same anterior and posterior graft surfaces). It gave significantly less inhibition than 12/2345 (same length, anterior graft surface moved forward). The length hypothesis is ruled out and a gradient of inhibition in the slug is suggested. The figures in columns 2 and 3 are results of separate experiments.

segment. According to the first hypothesis, this result would be impossible. The prediction was tested and found true (Table 3).

The present data show that tip regulation in the *Dd* slug can be described in terms identical to those used to describe hypostome regulation in hydra. Results obtained with both organisms are well explained by assuming interactions between a fast diffusing substance (I or *r*)^{8,13} and a non-diffusible or slowly diffusing substance or cell-state variable (P or *a*). In this respect, both hydra and the slug resemble a third pattern-forming system (the insect segment)¹⁴. These similarities suggest the existence of a general class of pattern-forming system. They indicate that insights into *Dd* pattern formation will be of general interest.

For clarity, and to emphasise similarities, I have discussed the data in terms of Wolpert's model for the regulation of hypostome formation in hydra I note that the results obtained

Table 2 Inhibition by normal and decapitated segments

Graft	Fraction of secondary axes inhibited	No. of grafts	Mean fraction of inhibition
12/2345	0.77	13	0.74
	0.60	10	
	0.60	10	
	0.73	15	
	0.60	10	
	0.80	10	
	0.79	14	
	0.88	8	
	0.88	8	
	0.75	8	
2/2345	1.00	11	0.83
	0.88	8	
	0.63	8	
	0.88	8	
	0.75	8	
	0.75	8	
1234/45	0.88	8	0.77
	0.67	12	
	0.92	12	
	0.88	8	
	0.75	8	
	0.58	12	
34/45	0.82	11	0.75
	0.77	9	
	0.75	8	
	0.63	8	
	0.83	8	
4/45	1.0	9	0.84
	0.88	8	
	0.80	10	
	0.70	10	
	0.80	8	

Tipless slug segments, and segments lacking longer anterior portions (12, 123) were compared with their intact homologues as inhibitors of secondary axis formation. None of these decapitated segments seems less effective than their homologues as inhibitors of secondary axis formation. Grafts leading to close proximity between the fronts of anterior and posterior segments gave slightly increased inhibition (2/2345 gives significantly more inhibition than 12/2345). The figures in columns 2 and 3 are results of separate experiments.

in hydra and *Dd* can be explained by at least one other type of model based on two variables¹⁵. There is not yet any evidence to justify commitment to a particular model.

The finding that there is an axial gradient in the capacity of a slug tip to inhibit formation of a second tip must also be evaluated in terms of what is already known about the tip as an embryonic organiser.

The tip appears first in the late *Dd* aggregate^{6,9}. It is then an organiser because it is a pacemaker region, where waves of chemotactant secretion are initiated. The tip thus controls the movements of the aggregating cells. The waves have polarity (that is, propagate centrifugally), but, as far as is known,

Table 3 Fate of anterior tissue in normal and decapitated chimaeras

Graft	Fractions of grafts in which			No. of grafts
	Front tissue stays at front	Mixture of front and back tissue at front	Front tissue moves back	
12/2345	1.00	0.0	0.0	50
2/2345	0.51	1.11	0.38	47

Hypothesis 2 predicts that grafts of tipless anterior segments may give rise to chimaeric slugs in which the tip comes from the posterior segment. Grafts using tipped anterior segments should never do this. To test the prediction, we made 12/2345 and 2/2345 grafts, using a (neutral red) stained anterior segment. In 12/2345 grafts, the stained tissue always remained at the front of the chimaeric slug (there is some diffusion of dye from the stained part). In 2/2345 grafts, the whole stained piece moved backwards in 38% of cases. The slug tip was then made from white (posterior) tissue. The prediction is vindicated. The frequent posterior tip formation observed in the 2/2345 grafts implies that competition between anterior and posterior developing tips is almost unbiased in this graft. This is consistent with the strongest form of hypothesis 2 (tip as sole source of inhibitor, with little inhibition by a decapitated 2 region).

convey no scalar information. Their effectiveness as signals does not diminish with distance (r) from the tip^{3,4,15}.

The ability of an aggregate tip to inhibit formation of a neighbouring tip should work by way of entrainment of the presumptive tip tissue and its prospective field by these propagating waves. This inhibitory effect should thus be independent of r . The finding of a distance dependent inhibitory effect in the slug suggests that the slug tip functions differently than the aggregate tip. I suspect that this difference in function relates to the genesis of a regulative scalar pattern of differentiation in the slug^{16,17}. The relevance of these findings to previous models for *Dd* pattern formation is discussed elsewhere (in preparation).

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Diet-induced alterations in distribution of multiple forms of alcohol dehydrogenase in *Drosophila*

It is now well accepted that enzyme polymorphisms are widespread among organisms. Electrophoretic polymorphisms in particular have been extensively studied and discussed¹. One enzyme, alcohol dehydrogenase (ADH) has received special attention because the relative proportions of two polymorphic forms (allozymes), *Adh^F* and *Adh^S*, in *Drosophila*, seem to vary with environmental temperature^{2,3}. The situation with regard to *Drosophila* ADH, however, is complicated by the fact that even in flies homozygous for a given electrophoretic variant or allozyme, three forms of ADH activity are observed after electrophoresis^{4–6}. They are distributed in such a way that (on agar gel electrophoresis) the one which migrates most cathodally (termed *ADH⁵* in the *Adh^F* strain) has the highest activity, but is the least stable to heat. The least cathodally migrating form (*ADH¹* in the *Adh^F* strain) has the least activity and is the most heat stable, whereas the third form (*ADH³* in the *Adh^F* strain) is intermediate with respect to both activity and heat stability (Fig. 1*a*). These forms are termed isozymes, in order to contrast them with the allozymes described above.

We have been interested in ADH and its genes because of their potential utility in investigating gene control in *Drosophila*^{7–10}. We have formulated a model to explain the mechanism of formation of the isozymes¹¹, according to which, the three isozymes in any one homozygous strain have the same primary structure: they are dimers of identical subunits of molecular weight 2.5×10^4 (ref. 11). The different electrophoretic mobilities of the three isozymes result from the differential, non-covalent binding of a low molecular weight, negatively charged molecule. *ADH⁵* has none of this molecule

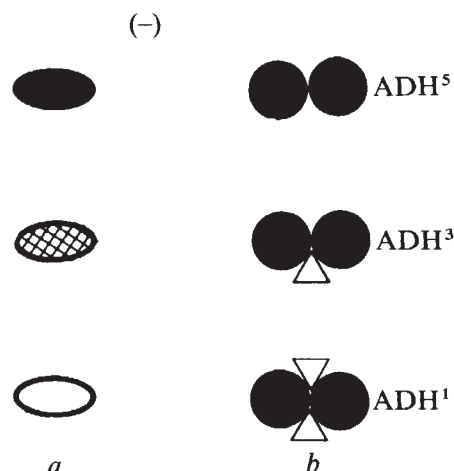


Fig. 1 Schematic representation of the isozymes of *Adh^F*. *a*, Migration is towards the cathode. The most intense strain is represented by a solid oval, intermediate staining by the cross-hatched oval, and the open oval represents the least intensely stained zone. *b*, Model to explain the formation of the three isozymes. The circles represent the subunits of ADH, and the triangles a negatively charged, NAD-carbonyl compound addition complex bound non-covalently to the dimer. *ADH⁵* has one mol of this compound bound; *ADH³*, two mol (ref. 11 and J. O'Donnell, M. S. and W. S. unpublished).

bound, but *ADH³* and *ADH¹* have one and two mol bound per mol of enzyme (Fig. 1*b*). The low molecular weight molecule has been tentatively identified as an addition complex of nicotinamide adenine dinucleotide (*NAD⁺*) and a carbonyl compound (ref. 11 and J. O'Donnell, M. S. and W. S. unpublished) of uncertain structure. (In contrast to the epigenetic mode of formation of the isozymes, the allozymes are thought to display differences in electrophoretic migration because of changes in the primary structure of the ADH protein.)

This model makes some very specific predictions. One is that if the concentration of the addition complex is increased, the relative proportion of the isozymes should change. Specifically (in the *Adh^F* strain), *ADH¹* and *ADH³* should form at the expense of *ADH⁵*. We therefore fed *Adh^F* flies a number of compounds that are known to form addition complexes with *NAD⁺* (ref. 12). A variety of other compounds, substrates of ADH, and some related compounds were also fed. An example of the results of these experiments is shown in Fig. 2. In this experiment, 3-hydroxy-2-butanone (acetoin) was fed to adult flies for 24 h in various concentrations. There is a decrease in

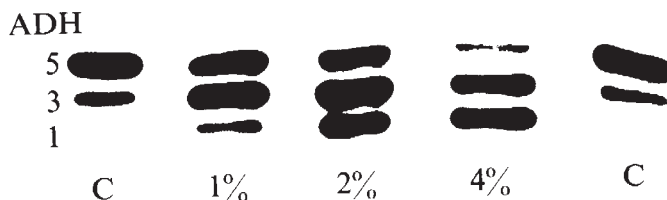


Fig. 2 Effect of acetoin in fly food on the isozymes of ADH. Acetoin was dissolved in water at concentrations indicated in the figure. (Controls, C, contained no added acetoin.) The solutions were added to four separate 2.6×8 -cm vials containing dry Instant *Drosophila* Medium (Carolina Biologicals). Fifty adult (> 4 d old) *Adh^F* flies were transferred to each of the vials and incubated at 25 °C for 24 h. The vials were sealed with a layer of Parafilm. After incubation the flies were collected, homogenised in 1 ml of 0.02 M sodium phosphate buffer, pH 7.5, and centrifuged at 12,000*g* for 15 min. The supernatant fluids (SFs) from these flies were then used for agar gel electrophoresis according to the procedure of Ursprung and Leone⁴. The gels were stained for 1 h at 29 °C for ADH activity using the staining procedure of Vigue and Sofer¹⁷. The positions of migration of *ADH⁵*, *ADH³* and *ADH¹* are shown at the left of the figure. (The photograph has been processed by a high contrast procedure in order to show the results more clearly.)

the apparent activity of ADH⁵ and a concomitant increase in activity of ADH³ and ADH¹ as a function of increasing concentrations of acetoin. (With an increased period of exposure, all the ADH activity can be shifted to the ADH¹ position.) In similar experiments, the following compounds were also found to lower the apparent activity of ADH⁵ and raise that of ADH³ and ADH¹: 2,3-butanedione, 2,3-butanediol, 1,3-butanediol, 2-propanone (acetone), 2-butanone, 2-butanol and 2-propanol (all at 1% concentration). All these compounds possess a secondary alcohol or carbonyl group, which correlates well with the marked substrate preference of *Drosophila* ADH for secondary alcohols⁴.

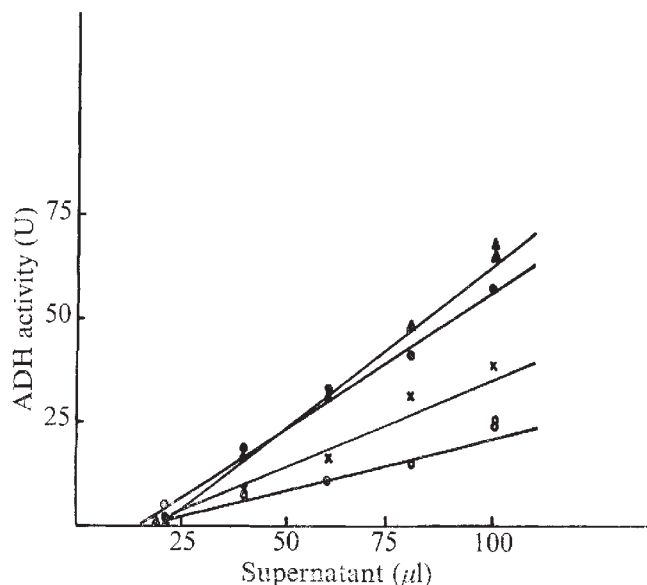


Fig. 3 Measurement of total number of ADH molecules by immunological titration of the fly supernatant fluids (SF, described in Fig. 2) shown in Fig. 2 with anti-ADH serum. Increasing amounts of fly homogenates were added to a constant volume of anti-ADH serum (prepared as described previously¹⁰). The abscissa indicates the volume in μ l of the SF added to a constant volume (20 μ l) of antiserum. After incubation for 2.0 h at 25 °C and 0.5 h at 4 °C, the mixtures were centrifuged at 12,000g for 20 min. The activity (in U per 50 μ l) remaining after centrifugation is plotted as a function of μ l of the original SF added. Δ , SF from control (0% acetoin) flies added to 20 μ l of antiserum; $\bullet$, SF from flies subjected to 1% acetoin for 24 h added to 20 μ l of antiserum; $\times$, SF from flies subjected to 2% acetoin for 24 h added to 20 μ l of antiserum; $\circ$, SF from flies subjected to 4% acetoin for 24 h added to 20 μ l of antiserum. The slopes of the four curves obtained after a linear regression analysis of the points are 0.81, 0.66, 0.50, 0.27 U per μ l of the control, 1%, 2% and 4% of SFs respectively. The x intercept, the equivalence point, is a function of the quantity of ADH-cross-reacting material. On the other hand, the slope of the line after the equivalence point represents the ADH activity per fly. Although the slopes decline threefold as a function of increasing concentrations of acetoin in the food, the equivalence points remain about the same. The equivalence points are 20, 13, 21, and 14 μ l respectively.

No significant alteration in the proportions of isozymes was found after feeding with 1% butyraldehyde, *n*-butanol, pyruvic acid, glyoxal, 1,5-pentanediol, ethanol, 1-propanol or acetaldehyde. In addition, the following compounds were fed (at 1% concentration) and were found to be lethal; cyclohexanone, cyclohexanol and 2-pentanone. Many of the other compounds caused varying degrees of mortality, but enough flies remained alive for electrophoretic analysis. No simple relationship was observed between mortality and the ability to alter the ratios of the isozymes.

The changes observed after feeding acetoin and other effective compounds are termed "conversion." This term implies that either molecules originally destined to become ADH⁵, form ADH³ and ADH¹, or that ADH⁵ molecules change into ADH³ and ADH¹ molecules. In either case, the total ADH

activity would be expected to drop (since ADH³ and ADH¹ have lower specific activities than ADH⁵) as a result of conversion, but the total number of molecules of ADH in all forms would remain the same. The data in Fig. 3 confirm our predictions and support the interpretation that conversion is taking place.

Two other predictions of the model have been confirmed. First, since strains homozygous for the three known allozymes each display three isozyme zones after electrophoresis, it is expected that each would show a similar response to being fed the effective compounds. Indeed, we found that in strains homozygous for the Adh^P and Adh^S variants, there was a change in the proportions of the three isozymes such that the less cathodally migrating forms seemed to increase in activity, whereas the more cathodally migrating forms lost activity after addition of acetoin to the food.

Second, since it was postulated that the isozymes originated from changes in ADH and not some other related enzyme, no bands should appear after the feeding of effective compounds in strains lacking ADH activity. In fact, no ADH activity was detectable after electrophoresis after feeding flies of the Adh⁰¹ strain 1% acetoin for 24 h.

Finally, we reared flies on sterile Sang's synthetic medium¹⁴ to test the hypothesis that microbial flora were producing some compound that was causing the normal appearance of the isozymes. No differences in relative intensity of the isozymes were observed, however, when sterile cultures were compared with cultures kept in Sang's medium with added live yeast.

The experiments reported here are important for two reasons. First, since we predicted that some compounds that are capable of forming addition complexes with NAD⁺ would alter the distribution of the isozymes of ADH, the data lend additional support to our model for the origin of the multiple forms of ADH. Second, these studies show that the electrophoretic migration of ADH can be changed greatly by events presumably occurring outside the *Adh* locus. It is conceivable that mutations may arise which affect the metabolism of these carbonyl compounds such that their concentration increases, resulting in changes in migration of ADH on electrophoresis. Thus, electrophoretic variants may occur in ADH with no change in the primary structure of the enzyme. For population geneticists working with this and similar enzymes, it would seem advisable, where possible, to carry out a genetic analysis of any new variants to show that the polymorphism maps close to the structural locus of the enzyme.

The three isozymes also differ significantly in stability¹⁵, and any alteration in the ratios of activity of the three forms could cause a shift in the heat stability of total ADH extracted from flies. Since it has been reported that natural populations are heterogeneous with respect to the heat lability of some enzymes¹⁶, it would be reasonable in the case of ADH and related enzymes to check for differences in the activity ratios of the isozymes. Here again mutations at loci outside of *Adh* which affect the amount of NAD or carbonyl compound could very well influence the stability of the enzymes without affecting its primary structure.

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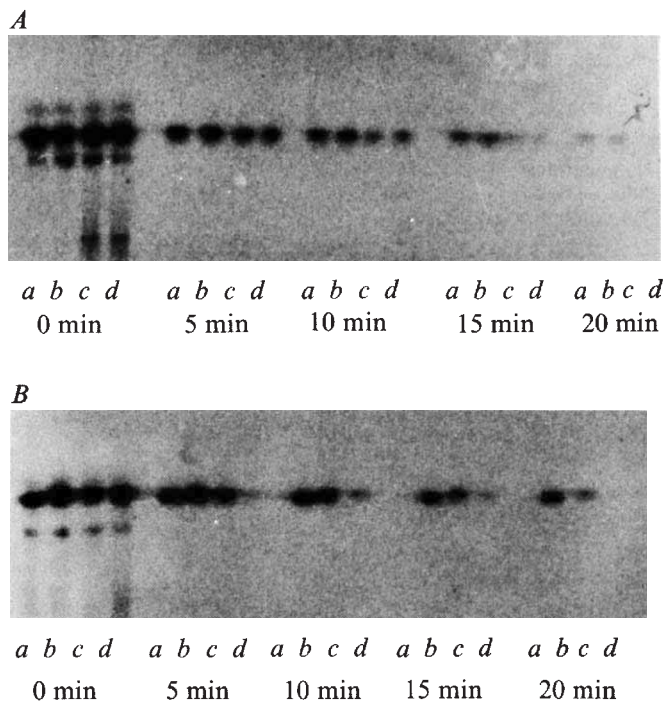


Fig. 1 Heat-sensitivity profiles of *Est-6* alleles. *A*, *Est-6*^{1.00}; *a* and *b*, *Est-6*^{1.00-1}; *c* and *d*, *Est-6*^{1.00-2}; heated for the indicated times at 59.5°C. *B*, *Est-6*^{1.10} and *Est-6*^{1.10-1}; *a*, *Est-6*^{1.10}; *b*, *Est-6*^{1.10-1}; *c*, *Est-6*^{1.10-2}; *d*, *Est-6*^{1.10-3}; heated for the indicated times at 57°C.

to be unaffected by being heterozygous for a second electrophoretic variant.

Figure 1*A* is a zymogram showing the two heat-stability classes found among *Est-6*^{1.00} lines, while Fig. 1*B* shows the *Est-6*^{1.10} heat-stability classes and the allele, *Est-6*^{1.08}, mentioned above. The class designated *Est-6*^{1.10-3} is similar to the allele *Est-6*^{F1} reported by Wright and MacIntyre, while *Est-6*^{1.10-1} is probably identical to their *Est-6*^{F2} (ref. 14).

It is difficult to state with certainty that the observed variation is, indeed, due to variation at the esterase-6 structural locus. The following lines of evidence are, however, consistent with such an hypothesis. (1) *F*₁ progeny of the crosses *Est-6*^{1.00-1} × *Est-6*^{1.00-2}; *Est-6*^{1.08} × *Est-6*^{1.10-3}; *Est-6*^{1.10-1} × *Est-6*^{1.10-2}, and *Est-6*^{1.10-1} × *Est-6*^{1.10-3}, and their reciprocals show heat stabilities intermediate to those of the parental lines. (2) *F*₂ progeny of the crosses *Est-6*^{1.00-1} × *Est-6*^{1.00-2} and *Est-6*^{1.10-1} × *Est-6*^{1.10-3} show segregation for heat stability not significantly different from the 3:1 ratio of stable to labile bands expected if homozygotes of the more stable allele and heterozygotes are scored as stable, while homozygotes for the less stable allele are scored as labile.

The allele designated *Est-6*^{1.08} is exceptional. Preliminary data suggest that a second locus alters the electrophoretic mobility of the *Est-6*^{1.08} allele, such that it is indistinguishable from *Est-6*^{1.10}. This modifier locus seems to have two allelic variants, one of which is dominant and results in this alteration. But the presence of occasional double-banded phenotypes in *F*₂ progeny which are segregating for the modifier locus suggests that *Est-6*^{1.08} and *Est-6*^{1.10} lines differ at both the structural and

Heat stability variants of esterase-6 in *Drosophila melanogaster*

It has been widely recognised that electrophoretic mobility alone does not distinguish all allelic variants of enzymes that exist in natural populations¹⁻⁴. Recent applications of various techniques, among them examination of the heat stability of allozymes, have revealed the existence of allelic variants among allozymes with identical electrophoretic mobility⁵⁻⁸. I report here the existence and frequencies of heat-stability variants of esterase-6 in populations of *Drosophila melanogaster*, as well as an electrophoretic variant whose mobility is altered by some other, probably linked, modifier locus.

Two common alleles of esterase-6 exist in natural populations as a stable polymorphism. In addition, several rare electrophoretic variants, null alleles and heat-stability alleles have been reported⁹⁻¹⁵. The nomenclature of Franklin¹⁵ has been used for esterase-6 alleles. My results show that, in the populations sampled, *Est-6*^{1.00} is a class of two alleles which can be differentiated on the basis of heat-stability, while *Est-6*^{1.10} is a class composed of three such alleles. Finally, *Est-6*^{1.08} has a mobility intermediate to those of *Est-6*^{1.00} and *Est-6*^{1.10} when homozygous but it is indistinguishable from *Est-6*^{1.10} when heterozygous with all other lines tested (unpublished data).

Males caught in the wild were crossed individually to females heterozygous for the marked balancer chromosome TM3¹⁶, and a series of lines homozygous for a single third chromosome were obtained through the usual series of crosses¹⁷. The esterase-6 locus is located at 36.8 on the third chromosome⁹. Twenty males from each line were pooled and homogenised in 0.2 ml of 0.1 M sodium phosphate buffer, pH 6.5. Aliquots of this crude homogenate were heated for 0, 5, 10, 15, and 20 min at previously determined temperatures (57.0°C for lines carrying *Est-6*^{1.10}, 59.5°C for lines carrying *Est-6*^{1.00}), in a constant temperature, circulating water bath with an accuracy of ±0.1°C. The control and heated aliquots were then subjected to starch gel electrophoresis according to the methods of Richmond¹⁸ and scored visually for staining intensity of the esterase-6 band.

Many of the extracted chromosomes carried recessive lethal genes and such lines could not be made homozygous. In these cases, if the lethal chromosome carried the *Est-6*^{1.10} allele, it could be examined in +/TM3 flies, for TM3 carries *Est-6*^{1.00}. Lethal chromosomes carrying *Est-6*^{1.00} could be likewise examined by outcrossing to *Est-6*^{1.10} homozygotes, since all wild-type progeny of such crosses would be genotypically *Est-6*^{1.00}/*Est-6*^{1.10}. Similar crosses involving non-lethal chromosomes, which facilitated comparison of homozygotes and *F*₁ heterozygotes, showed the heat stability of the relevant band

Table 1 Frequencies of *Est-6* alleles observed with electrophoretic and heat-stability criteria

Location	N*	Electrophoretic alleles				Heat-stability alleles within electrophoretic classes						
		1.00	1.08	1.10	1.15	1.00-1	1.00-2	1.08	1.10-1	1.10-2	1.10-3	1.15
Bloomington, Indiana 1	38	0.757	0.108	0.135	0.0	0.135	0.622	0.108	0.0	0.027	0.108	0.0
Bloomington, Indiana 2	40	0.650	0.075	0.250	0.025	0.0	0.650	0.075	0.100	0.050	0.100	0.025
McDougall, New York	22	0.682	0.0	0.318	0.0	0.091	0.591	0.0	0.227	0.0	0.091	0.0
Tempe, Arizona	8	0.625	0.0	0.375	0.0	0.0	0.625	0.0	0.125	0.125	0.125	0.0

*Number of chromosomes sampled.

Table 2 Latter indices of genetic distance, based on frequencies given in Table 1

Location 1	Location 2	Electrophoretic alleles			Electrophoretic and heat-stability alleles		
		δ_B	δ	$\delta_B - \delta$	δ_B	δ	$\delta_B - \delta$
Bloomington 1	Bloomington 2	0.396	0.378	0.018	0.505	0.478	0.027
Bloomington 1	McDougall	0.376	0.340	0.036	0.531	0.481	0.054
Bloomington 1	Tempe	0.406	0.350	0.056	0.526	0.477	0.049
Bloomington 2	McDougall	0.387	0.378	0.009	0.506	0.476	0.030
Bloomington 2	Tempe	0.402	0.387	0.015	0.484	0.472	0.012
McDougall	Tempe	0.353	0.349	0.004	0.506	0.476	0.030
Mean		0.387	0.364	0.023	0.510	0.477	0.034
Standard error		0.008	0.008	0.008	0.007	0.001	0.006

the modifier locus. Thus, for the purpose of examining gene frequencies at this locus, I have considered *Est-6*^{1,8} to be an electrophoretic allele.

Table 1 gives the observed allele frequencies among extracted lines for the esterase-6 locus, according to geographic origin, when mobility alone, and mobility and heat stability are considered. With the exception of *Est-6*^{1,15}, which occurred only once, no allele is unique to a population, although *Est-6*^{1,8} was found only in the Bloomington, Indiana area. This is in contrast to the results reported by Bernstein *et al.*⁵ in which two neighbouring populations of *D. a. americana*, which shared three of four electrophoretic alleles, were found to share only one of nine stability alleles. No such radical differences in the genetic similarities of populations compared were observed in the system under consideration here.

Latter¹⁹ has proposed a measure of genetic distance between populations that is suitable for the comparison of closely related populations whose hierarchical relationship is not known. He defines two quantities: δ , a measure of average genetic distance between individuals within populations, and δ_B , a measure of genetic distance between populations. They are defined as follows

$$\delta = 1 - \frac{1}{2} \sum_i (p_{i1}^2 + p_{i2}^2) - \frac{1}{2} \sum_i [p_{i1}^2 (1 - p_{i1})^2 + p_{i2}^2 (1 - p_{i2})^2] \quad (1)$$

$$\delta_B = 1 - \sum_i p_{i1} p_{i2} - \sum_i [p_{i1} (1 - p_{i1})] [p_{i2} (1 - p_{i2})] \quad (2)$$

where p_{ij} is the frequency of the i th allele in the j th population. The net difference between populations is thus $\delta_B - \delta$.

Values for all pairwise comparisons of the populations sampled are given in Table 2, using both electrophoretic and heat stability classes. While application of the second criterion does increase δ and δ_B , the average net difference between populations is not increased significantly. Thus, the pattern of relative constancy of allele frequencies reported by O'Brien and MacIntyre¹⁰ on the basis of electrophoretic mobility alone is not significantly altered by application of the heat stability criterion.

Genetic distance estimates are usually based on an averaging of several loci in populations¹⁹. However, Hedrick²⁰ has pointed out that if selective forces vary from locus to locus, such averaging will tend to give deceptive results. Therefore, the use of such an approach at the one locus studied here is valid as a measure of population differences at that locus.

Analysis of the reported data does not follow the predictions of Maynard Smith³ and King⁴, who propose that such variation is selectively neutral, and that the geographically uniform patterns of gene frequencies observed are a result of the limited number of charge states which the protein can assume. If such were the case, frequencies of hidden alleles should show a more random distribution than those of electrophoretic alleles. In the case of esterase-6 in *D. melanogaster*, no significant increase in randomness of gene frequencies is evident. This fact and these data are not sufficient, however, to indicate the action of selection at this locus.

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Method to test inhibitory antibodies in human sera to wild populations of *Plasmodium falciparum*

PROTECTIVE antibodies are involved in acquired immunity to malaria parasites¹. Sera from monkeys immune to *Plasmodium knowlesi* contain antibodies which depress multiplication of the parasite *in vitro*² by, it is believed, inhibiting the invasion of red cells by merozoites^{3,4}. Antibodies inhibitory to parasites of a specific antigenic variant are of a higher titre than inhibitory antibodies which cross-react with different antigenic variants⁵. We have attempted to extend these findings to natural human infections with *P. falciparum* in The Gambia, West Africa, where *P. falciparum* malaria is hyperendemic. The entire population is exposed to infection and an effective immunity is only acquired over 4-5 yr (ref. 6). Immunity to *P. falciparum* can be passively transferred with IgG from immune Gambian adults⁷.

Gambian sera were screened *in vitro* for anti-*P. falciparum* activity as follows. Ring-stage parasites obtained from infected children by venepuncture were grown through schizogony in microtissue cultures⁸ containing in replicate 150 μ l of supplemented medium TC-199 and 100 μ l of the following sera: (1) serum from the donor of the infected red cells; (2) Caucasian AB serum collected in the United Kingdom, and (3) serum from unrelated Gambians (matched for the ABO blood group of the infected cell donor). Parasite growth after reinvasion was measured by

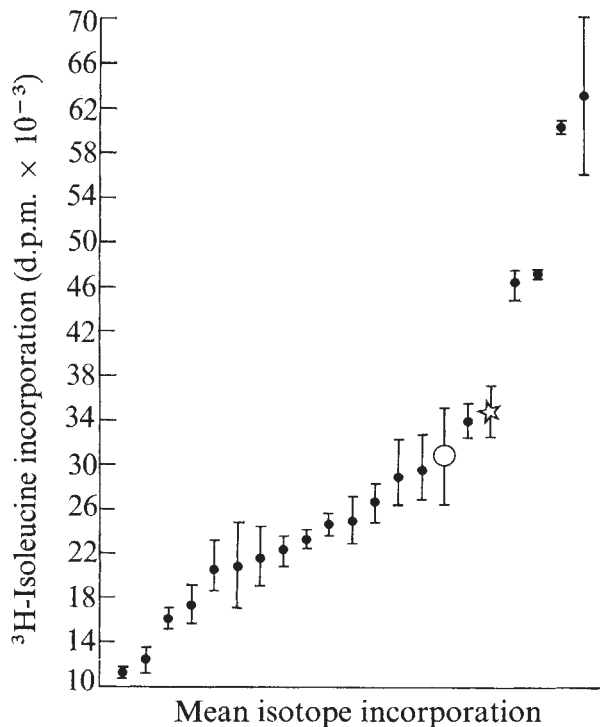


Fig. 1 Incorporation of ^3H -isoleucine by *P. falciparum* parasites grown through schizogony *in vitro* in the presence of sera from 21 different donors. Isotope (specific activity 26 Ci mmol $^{-1}$) was added after schizogony to give 1 μCi per culture well. Cultures were collected 43 h later. Star, autologous serum; $\circ$, Caucasian AB serum; $\bullet$, African adult serum. Bars represent range of values.

incorporation of ^3H -isoleucine and parasite multiplication was assessed by microscopic examination of smears of the cultured blood stained with Giemsa stain.

In Fig. 1, the inhibitory effects on parasite growth of sera from 19 immune African adults are compared with the effects of two control sera. Most of the sera from African adults inhibited parasite multiplication and hence the incorporation of isotope in the ensuing growth period. In a few (4/19) sera, parasite multiplication and isotope incorporation were enhanced compared with controls. More than 100 sera from Gambians aged 1–65 yr have been tested with parasites isolated from many different children. In about 71% of the sera the multiplication rate of the parasites was at least 10% less than that in serum from the donor of the parasites. But multiplication was inhibited by 20% or more in only 61% of the sera tested and by 50% or more in only 31% of the sera tested. Besides this rather low degree of inhibition, variability in the amount of inhibition was a frequent feature when the same sera were tested against parasites from different children. Table 1 summarises data from an experiment in which 11 sera from Gambian adults were tested against parasites isolated from four different infected children; the individual sera inhibited parasites from some children but not from others.

These findings raise questions about antigenic diversity in *P. falciparum* parasites, as strong inhibitory activity might depend on correct matching of parasite (antigenic) type and antiserum. In addition, the specific inhibitory antibody response might be short lived, like the precipitating antibody response to certain *P. falciparum* antigens⁹. Matching of parasite population to antiserum was not controlled and there was no knowledge of the temporal relationship between them in the experiments discussed above, nor was there in the studies of Phillips *et al.*¹⁰ and Mitchell *et al.*¹¹. The remainder of this report concerns a method we have

devised to test known homologous or heterologous combinations of parasitised cells and sera.

Heparinised blood (17 U ml $^{-1}$), as well as serum, was obtained from infected children. The blood was cryopreserved in a final concentration of 10% glycerol in Ringer solution at -20°C ; the serum was also frozen. Each child was treated once with chloroquine (5 mg base per kg) immediately after the infected blood was collected and a second serum sample was obtained 1–3 weeks later, after catabolism of the antimalarial. When the second serum sample was collected, 43 of the 50 donor children no longer had a patent asexual parasitaemia. It was expected that in the interval between collection of the two serum samples, each child would develop a specific immune response to the parasite population of which a representative sample had been cryopreserved. Precipitating antibodies usually appear within a week or two of treatment¹². To compare the inhibitory activities of the first (I) and second (II) serum samples, the appropriate frozen parasites were reconstituted and grown through schizogony *in vitro* in the presence of known homologous or heterologous sera.

Table 1 Distribution of 11 adult sera according to the degree of inhibition they produced when tested with four different isolates of *P. falciparum*

Percentage inhibition of parasite multiplication	1	2	3	4
None	3	0	11	1
Up to 20	5	1	0	4
Up to 40	3	2	0	5
Up to 60	0	8	0	1

Studies on the metabolism¹³ of chloroquine in man indicate that the dose we administered should have reached the minimum therapeutic level by day 7, the earliest time that we obtained the second serum sample from any child. To check for any antimalarial activity through residual chloroquine, we carried out cytotoxic tests with the sera in micro-tissue cultures using *P. knowlesi* as a model system. The results indicated that there was an overall reduction in the uptake of ^3H -isoleucine by *P. knowlesi* parasites grown in serum (II) samples, but this was not significant at the 5% level. The few (5/31) serum (II) samples that had a marked inhibitory effect on *P. knowlesi* did not consistently inhibit *P. falciparum*, nor were they among the samples taken

Table 2 *Plasmodium falciparum* multiplication in serum obtained before (I) and after (II) antimalarial treatment

System	Serum	cells	(I)	(II)
Homologous	7529	7529	100	(12) 19
	7536	7536	100	(14) 11
	7540	7540	100	(14) 45
	7544	7544	100	(14) 219
Heterologous	7519	7529	102	(14) 20
	7523	7529	52	(14) 28
	7525	7529	42	(7) 26
	7519	7536	56	(14) 2
	7529	7536	113	(12) 4
	7519	7539	136	(14) 56
	7523	7539	133	(14) 122
	7525	7539	96	(7) 67
	7529	7539	157	(12) 67
	7536	7539	135	(14) 102
	7506	7540	52	(14) 17
	7531	7540	146	(14) 108

Results are expressed as percentage of multiplication in homologous serum (I). Triplicate tissue cultures each contained 10 μl of blood + 150 μl TC-199 + 100 μl of serum. Percentage multiplication was calculated from the mean results. Italic figures indicate number of days between antimalarial treatment and collection of serum (II).

Table 3 Uptake of ^3H -isoleucine (c.p.m. $\times 10^{-3}$) by *P. falciparum* grown in serum obtained before (I) and after (II, III) antimalarial treatment

System	Serum	Cells	AB	I	II	III
Homologous	7525	7525	1.31	3.21	(7) 0.23	(24) 2.09
	7531	7531	ND	3.32	(14) 0.05	
	7544	7544	2.25	5.98	(14) 12.84	
	7580	7580	1.46	2.54	(7) 0.28	
	7584	7584	1.76	2.19	(7) 0.73	
Heterologous	7569	7584	1.76	4.13	(13) 1.08	

Duplicate tissue cultures each contained 10 μl of blood + 150 μl of medium TC-199 + 50 μl of serum + 10 μl of ^3H -isoleucine (1 μCi per well). Incorporation values are given as means. Italic figures indicate number of days between antimalarial treatment and collection of serum (II) or (III). ND, Not done; AB, Caucasian AB serum.

only 7 d after antimalarial treatment. Factors other than residual antimalarial may account for this inhibitory activity. Furthermore, a few children returned 2–3 weeks after chloroquine treatment, with patent parasitaemias and in these children there presumably was no persistent chloroquine activity.

Data on multiplication of *P. falciparum* parasites in children's sera taken before (I) and after (II) antimalarial treatment are shown in Table 2. Parasite multiplication is given as a percentage of that in homologous (I) serum, so figures greater than 100% indicate enhanced parasite growth in homologous (II) or heterologous sera.

Marked inhibition of parasite multiplication occurred in three of the four examples shown of homologous serum-cell mixtures. In the fourth example (7544) parasite multiplication was enhanced in homologous (II) serum (see below). Table 2 also shows results obtained with four isolates of infected cells that were tested against heterologous sera. Again there was marked inhibition of parasite multiplication in most of the serum (II) samples (*t* test for 12 paired (non-independent) samples gave $P < 0.001$). On the other hand, in heterologous (I) samples, inhibition occurred in only 4/12 cases (some cases of cross inhibition might be expected because all the children had been exposed previously to repeated malarial infection). The inhibitory activity of serum 7536 (II) in the homologous but not in the heterologous system (7539 cells) is notable. Specific inhibition is also suggested in the heterologous tests of serum 7523 (II) with cells from 7529 (inhibition) and 7539 (no inhibition).

Measurements of ^3H -isoleucine incorporation gave similar results. Table 3 shows that serum 7525 (III) taken 24 d after antimalarial treatment was less inhibitory than serum (II) obtained 7 d after treatment. Serum (II), however, was not inhibitory in the *P. knowlesi* cytotoxic assay. By contrast, serum 7544 (II) was inhibitory in the *P. knowlesi* assay but enhanced both parasite multiplication (Table 2) and isotope incorporation (Table 3) in homologous tests with *P. falciparum*. No explanation for this enhanced growth can be offered at present but it may possibly be a nutritional effect.

In conclusion, our results indicate that in most cases there was significant inhibition of parasite multiplication by children's convalescent sera. Both 'cross reactive' and specific inhibition reactions were observed. The possible roles of anti-parasitic antibodies, autoantibodies to red cells, and non-antibody effects in this system require further investigation, as does the blocking effect of malarial antigen released into the serum during infection or added experimentally.

Studies of their antigens¹⁴ and isozymes¹⁵ suggest that wild populations of *P. falciparum* parasites frequently consist of mixed types. At present there are no reliable methods either to measure the extent of this heterogeneity or to select components from it. The protocol we present here circumvents this problem and makes possible a test of the homologous inhibitory antibody response to a pure or mixed

population of parasites. The methods that we used to cryopreserve and reconstitute parasitised erythrocytes were unrefined but further work using 10% DMSO as the cryoprotectant has improved this aspect of the test. Consequently we feel that our method offers a practical approach to the analysis of anti-parasitic antibodies in human malaria infections. Cryopreservation of *P. falciparum*¹⁶ can be used to look for other functional antimalarial immune responses, such as cell-mediated cytotoxic responses¹⁷, directed against specific populations of the parasite at a particular time, and also to provide laboratories outside the tropical regions with infected blood, for example for *in vitro* drug screening.

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Possible repair of carcinogenic damage caused by dimethylnitrosamine in rat kidney

SINCE the discovery that cells from patients with xeroderma pigmentosum, a disease associated with a high incidence of cancer, may be unable to excise ultraviolet light-induced thymine dimers from DNA¹, and the recognition of the possibility that all carcinogens react with the DNA of the organs in which tumours are induced², it has been thought that repair of changes produced in DNA by carcinogens

might be important in moderating the effectiveness of these compounds. Work on the carcinogenic effect of prolonged administration of various chemicals³, however, suggests that individual carcinogenic doses are entirely cumulative in their action, and that the carcinogenic damage is permanent and not repaired⁴. If this were correct, repair of lesions produced in DNA by carcinogens would seem to have no direct relevance to carcinogenesis. Recovery from the lethal effects of radiation was established by the classic experiments of Elkind and Sutton⁵ in which cultured cells were irradiated twice with different intervals between the first and second dose; if sufficient time elapsed between the first and second dose, the effects of the doses were not cumulative. We have used an analogous experimental design to study the cumulation of the effect of dimethylnitrosamine in the induction of kidney tumours in the rat. The experiment is not yet complete, but we believe we have demonstrated that a repair process exists.

A single dose of dimethylnitrosamine induces cancer in the rat kidney. When rats are fed a protein-free, high-carbohydrate diet for 7 d before receiving the dimethylnitrosamine they will tolerate a single dose which will induce cancer in every rat⁶. A few tumours are induced in other organs, mainly in the lung, but the kidney tumours predominate and the other tumours do not kill the animal before the rats are old enough for any potential kidney tumour to have become detectable.

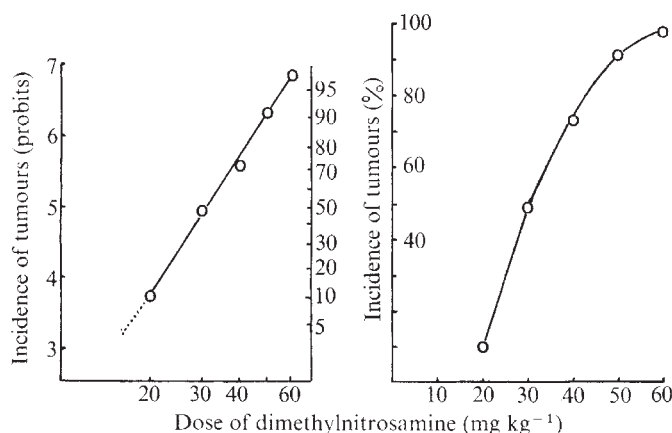


Fig. 1 Dose-response curve for the induction of kidney tumours 80 weeks after a single administration of dimethylnitrosamine. Rats were obtained from the same source and fed the same diet as in the experiment described in the legend to Fig. 2. Results are shown as *a*, incidence of tumours against dose; *b*, as the probit of incidence against the log of the dose. *b* was used to predict the incidence which might have been expected if other doses had been given. It can be seen that the incidence predicted from a single dose of 32 mg kg⁻¹ (52%) and from 16 mg kg⁻¹ (5%) were very close to those obtained in the experiment shown in Fig. 2 (55% and 6%).

The dose-response curve for the induction of kidney tumours was determined in the USA between 1972 and 1974. From this curve (Fig. 1) it can be predicted that a single dose of 16 mg per kg body weight will induce kidney tumours in 5% of the rats 80 weeks after the dose was given, rising to 10% after 2 yr. In contrast 32 mg kg⁻¹ will induce a 52% incidence at 80 weeks rising to 80% after 2 yr. We have estimated the rate and extent of recovery from carcinogenic damage from the incidence of tumours produced by one dose of 16 mg kg⁻¹ followed after some time by another dose. If the two doses were to be given so close together that no recovery from the carcinogenic damage could occur, the two would be completely cumulative in their effect and would be expected to give the same tumour incidence as a single dose equal to the sum of the two doses, that is, 32 mg kg⁻¹ giving a 52% incidence at 80 weeks, or 80% at 2 yr. If, however, repair of the damage

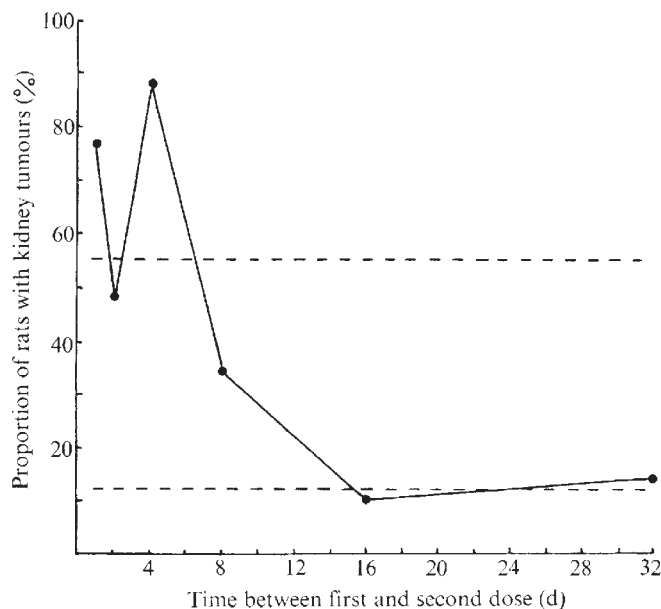


Fig. 2 Incidence of kidney tumours induced 80 weeks after a single intraperitoneal injection of dimethylnitrosamine (32 mg per kg body weight) (100 rats) a single dose of 16 mg kg⁻¹ (140 rats) or by two doses, the first of 16 mg per kg body weight, the second a "nominal" (see below) 16 mg kg⁻¹ 1, 2, 4, 8, 16 or 32 d later. (There were 50, 50, 60, 50, 100 and 100 rats in each group.) Male CFN rats (Carworth Farms, New City, New York) each weighing about 180 g were changed to a protein-free diet for 7 d, given an injection of dimethylnitrosamine, and then maintained on the same diet for 5 d. They were then returned to a normal rat diet (MRC 41B, Christopher Hill). The second dose was adjusted, as described in the text, so that the total amount of the kidney, when two doses were given, was believed to be the same as that resulting from a single dose of 32 mg kg⁻¹. This adjustment meant that the actual dose given (in mg kg⁻¹) 1 d after the first was 12; 2 d 13; 4 d 12.5; 8 d 26; 16 d 24 and 32 d 25. Eighty weeks after the first dose, tumours had been found in 212 of the 617 rats surviving more than 4 weeks. The upper dotted line shows the incidence produced by a single dose of 32 mg dimethylnitrosamine per kg body weight. If the doses were completely cumulative, one would expect two with the effect of 16 mg kg⁻¹ to give this incidence of tumours. A single dose of 16 mg kg⁻¹ gave an incidence of 6%. If the doses were not cumulative and each dose of 16 mg kg⁻¹ acted independently of the other, one would expect to get twice the incidence produced by a single dose of 16 mg kg⁻¹ (that is 12%) marked by the lower dotted line.

caused by the carcinogen occurs in the time between the first and second dose, a less than additive effect would be expected and, in the extreme case, when the two doses were separated by a sufficient interval, the tumour incidence would be that induced by each dose separately, that is, at 80 weeks 5%, plus 5% of the animals in which cancer was not induced by the first dose.

Dimethylnitrosamine requires metabolism for its carcinogenic activity, so the possible effect of the first dose on the metabolism of the second must be considered. The second dose must give the same extent of reaction with the critical target, as happens when the single dose is increased from 16 mg kg⁻¹ to 32 mg kg⁻¹. If the first dose of dimethylnitrosamine affects the metabolism of the second, the second dose required may be greater or less than 16 mg kg⁻¹. There is evidence that the extent of methylation produced in an organ by dimethylnitrosamine provides a measure of the extent of metabolism of dimethylnitrosamine in that organ, and of the extent of interaction of the ultimate carcinogen with the crucial cellular target⁶. By measuring the extent of methylation on the 7-position of guanine in the DNA of the kidneys produced by the second dose of dimethylnitrosamine, the second dose was adjusted so that, at every interval, the presumptive total extent of reaction with the

target cells was the same. The actual doses injected are given in the legend to Fig. 2.

The rats (in two batches) were given the first dose in April or May 1974. Of the 650 rats at the start of the experiment, 617 survived longer than 4 weeks. Of these, 437 are now dead, 259 of them with kidney tumours. The results (Fig. 2) seem to show that there is no repair (that is, the doses are at least cumulative) when the two doses are separated by no more than 4 d, but that apparently complete recovery takes place when the two doses are separated by 16 d or more. The reason for the considerable difference in incidence when the doses are separated by 1, 2 or 4 d is unknown. The steep dose-response curve magnifies small errors in dose to large differences in incidence, but the incidence when the doses were separated by 4 d is much greater than expected. The absolute amount of methylation of the *N*-7 and *O*⁶ position of guanine, and the effect of the first dose on the excision of the *O*⁶-methylguanine produced in the kidney DNA by the second dose, is being re-examined to try to explain it.

It is not likely that the recovery is the result of death of damaged cells and repopulation of the kidney by undamaged cells. Even 50 mg dimethylnitrosamine per kg body weight produces little necrosis in the kidney⁷, and the half life of the *N*-7-methylguanine produced by the carcinogen in DNA is approximately the same in the kidneys of rats given 16 mg kg⁻¹ as it is in the liver of rats given a dose which does not produce necrosis (ref. 8 and P. F. S. and R. Mace, unpublished). If there had been significant death and repopulation, the apparent half life of the alkylated base would have been shortened because the cells containing *N*-7-methylguanine would have been lost and replaced by cells which did not contain this alkylated base. The recovery therefore results not from repopulation but from repair of the critical damage which initiates the development of cancer.

The results reported here seem to be consistent with the hypothesis that the alkylation by dimethylnitrosamine and some alkylating agents of the *O*⁶-position of guanine in DNA is the critical chemical lesion in the induction of cancer by these compounds⁹ and that the animal can protect itself by excising this altered base from its DNA¹⁰⁻¹³. Although the rate and extent of the excision of dimethylnitrosamine-produced *O*⁶-methylguanine from the kidney DNA of rats given exactly the diet and dose we used has not been measured, Nicoll *et al.*¹² found that after a dose of dimethylnitrosamine, which would be the equivalent of 13 mg kg⁻¹ if the dietary conditions had been the same as ours, about 30% of the *O*⁶-methylguanine was excised from the DNA between 6 h and 15 h after the dose. No further excision took place from 15 h to 48-72 h, after which excision resumed. The block of excision parallels, and might explain, the lack of recovery from the carcinogenic effect during the first 4 d (Fig. 2) but it remains to be seen whether, when the larger dose is given to rats on a protein-deficient diet, the rate and extent of excision follow more exactly the recovery from the carcinogenic effect.

Our conclusion that carcinogenic damage can be repaired differs from that of Druckrey³, but the conclusion is not incompatible with his results, which were obtained from experiments in which carcinogen was administered repeatedly for long periods. It is not known what happens to the products of reaction between carcinogen and cell in these circumstances, but if the carcinogen acts by reacting with one of the bases in DNA so that its base-pairing properties are altered, the effect of this reaction product could be cumulative, either through accumulation of permanent base changes formed in the progeny cells during cell division, or through inhibition by the carcinogen of the excision from the DNA of the crucial reaction product. A large dose of dimethylnitrosamine inhibits the excision of *O*⁶-methylguanine from rat liver DNA¹⁴ and it is possible

that prolonged treatment with smaller amounts would have the same effect.

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Changes in the behaviour of teratocarcinoma cells cultivated *in vitro*

MOUSE teratocarcinoma cells have been recognised as important material for studying *in vitro* the processes of determination and differentiation that take place during normal embryogenesis^{1,2}. But in order for the system to be exploited maximally, it must be possible to manipulate at will the differentiation of mass cultures of pluripotent cells into endoderm, ectoderm and mesoderm derivatives. Martin and Evans showed that they could trigger the appearance of endoderm cells in undifferentiated cultures of the SIKR teratocarcinoma line by suspending aggregates of the cells in bacteriological Petri dishes to which they could not adhere^{3,4}. Within about 2 d the aggregates developed into simple embryoid bodies, resembling egg cylinders of normal embryos with an outer layer of endoderm cells surrounding a core of undifferentiated cells. In some SIKR clones, but not others, the inner cells later differentiate into neuroectoderm and mesoderm and the embryoid bodies develop fluid cysts resembling the visceral yolk sac vesicles of normal early embryos cultured *in vitro*. Not all lines of pluripotent teratocarcinoma cells, however, can be induced to form embryoid bodies in such a convenient way (ref. 5 and my observations). This could be because certain teratocarcinomas are for some reason intrinsically unable to form embryoid bodies, or because the cells can lose this ability during cultivation *in vitro*. In the course of isolating a new clonal line of teratocarcinoma cells I noticed that their ability to differentiate was strongly influenced by the way in which they were maintained *in vitro*. As long as the cells were grown on layers of feeder fibroblasts they retained the ability to form embryoid bodies but this was progressively

lost as the cells were adapted to grow on gelatin-coated dishes.

The clone used for this work came from an OTT 6050 ascites tumour derived by Dr L. C. Stevens from a 6-d strain 129/Sv mouse embryo grafted to the testis of an adult⁶. A frozen stock of the ascites tumour was acquired by Dr M. J. Evans of University College, London and went through a further six passages *in vivo* before being cloned in this laboratory. Embryoid bodies in the ascites fluid were trypsinised and dispersed into 9-cm dishes containing 3×10^6 STO mouse fibroblasts that had been treated before plating out with mitomycin C ($10 \mu\text{g ml}^{-1}$) for 3.5 h (ref. 3). When colonies of undifferentiated embryonal cells had grown, the plates were trypsinised and single teratocarcinoma cells were isolated as described by Martin and Evans³.

Cultures were maintained by plating approximately 5×10^4 cells into a 9-cm dish containing feeder fibroblasts in Dulbecco's modified Eagle's medium with 10% calf serum and 10% foetal calf serum (Flow Laboratories). The medium was changed daily and every 2–3 d the cells were detached with 0.05% trypsin and 0.6 mM EDTA in phosphate-buffered saline, pH 7.2, and subcultured. Until they were confluent, the cultures grew as homogeneous sheets of closely packed cells which spread evenly over the fibroblasts. Provided they were maintained in this way they kept their ability to differentiate into cystic embryoid bodies. The most efficient method of triggering differentiation was to suspend aggregates of the cells in bacteriological Petri dishes after a preliminary step to remove most of the feeder fibroblasts. The mixed cultures were trypsinised briefly to give clumps of 50–200 cells and these were plated out at low density on to a standard tissue culture dish in medium with 10% foetal calf serum. Overnight the fibroblasts attached tightly but the teratocarcinoma cells formed rounded aggregates which could be detached easily and transferred to bacteriological dishes. Two to three days after transfer, all aggregates resembled simple embryoid bodies: the outer endoderm cells had numerous vacuoles and microvilli, tight junctions and a well developed endoplasmic reticulum, and were separated from the inner core of undifferentiated cells by a thick basement membrane (analogous to the Reichert's membrane of normal embryos)³. By day 6 most of the bodies had developed a fluid-filled cyst and by 10 d about 20% of these contained small patches of haematopoietic cells.

Attempts to adapt the cells to grow in the absence of feeder fibroblasts did not succeed until (1) the dishes were coated with 1% gelatin (swine skin Type 1 300 bloom, from Sigma), (2) the cells were initially subcultured at high density and (3) the medium was supplemented with both 10% calf serum and 10% foetal calf serum. Even in these conditions, some cell death occurred during the first few subcultures, but eventually a morphologically homogeneous population of cells was obtained which grew rapidly (doubling time 20 h) in closely packed sheets and could be subcultured every 2 d at 1×10^6 cells per 9-cm dish. For the first few weeks these cells retained their ability to form cystic embryoid bodies when challenged by being put into bacteriological Petri dishes. With longer times in culture the cells would form only simple embryoid bodies. By 2 months (approximately 25 passages) only about 25% of the aggregates differentiated at all, whereas the remainder merely enlarged and became necrotic. By 4 months almost none of the clumps formed embryoid bodies after 5 d in suspension. These cultures were not entirely incapable of differentiating, however; if they were allowed to reach very high density on gelatin-coated dishes and the medium changed every day, scattered patches of endoderm cells appeared after about a week, followed by fibroblast-like cells and neurones, but this process was always accompanied by massive lysis of the undifferentiated cells. The loss of ability to differentiate rapidly seems to be irreversible

because it could not be restored by returning the deficient cells to feeder layers for several generations. The behaviour of these cells had then become very similar to that of other extensively studied OTT 6050-derived cell lines that were selected to grow in the absence of feeder fibroblasts on gelatin-coated⁷ or untreated dishes⁸.

These results suggest that during adaptation to growth on gelatin-coated dishes, strong selection occurs for cells which are better able to grow on a less adhesive substratum and perhaps because of this have a reduced probability of differentiating. Many of the advantages of using teratocarcinoma cells to study determination and differentiation are therefore lost if the cells are not maintained on feeder fibroblasts.

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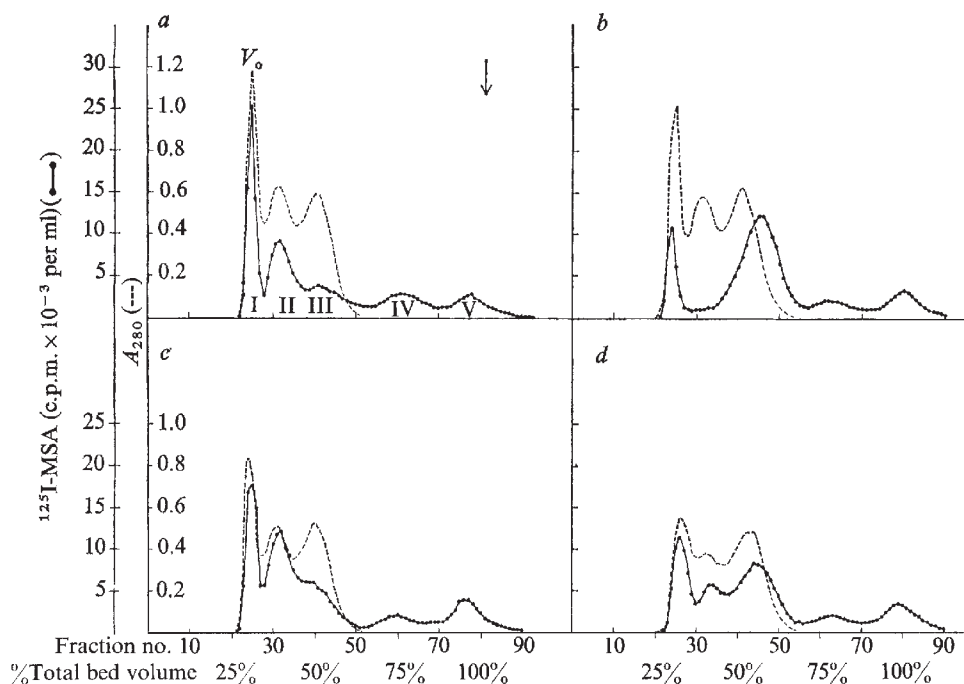
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Specific binding of a somatomedin-like polypeptide in rat serum depends on growth hormone

SOMATOMEDINS are growth hormone-dependent polypeptides that have been proposed as the mediators of the peripheral actions of growth hormone on skeletal tissue¹. Several polypeptides with somatomedin activity *in vitro*, including somatomedins A, B and C and non-suppressible insulin-like activity soluble in acid ethanol (NSILA-s)^{2–4}, have been isolated from human plasma. Unique among polypeptide hormones, the somatomedins circulate in human blood complexed to binding proteins of larger molecular weight^{2,4–7}. We report here the specific binding of a somatomedin-like polypeptide to distinct protein fractions in rat serum and further demonstrate that the pattern and level of this binding is dependent on growth hormone. We postulate that specific binding of somatomedin modulates the peripheral effects of somatomedin activity.

Results from our laboratory⁸ have suggested that somatomedin-binding proteins prolong the half life of somatomedin activity in the circulation. In those studies, the half life of somatomedin activity derived from the serum of growth hormone-treated hypophysectomised (Hx) rats and injected into recipient Hx rats was determined. The half life of somatomedin activity from Hx animals treated with growth hormone for 10 d approached the half life of somatomedin activity derived from normal rats (3–4 h) and was significantly longer than the half life (< 15 min) of somatomedin activity derived from Hx rats treated with growth hormone for only 24 h. Furthermore, the half life of somatomedin activity that had been dissociated from serum proteins of large molecular weight by boiling in acid conditions or by chromatography on Sephadex G-50 in acid conditions before injection into Hx animals was less than 30 min. If this low molecular weight somatomedin activity was recombined with the serum proteins of high molecular weight from which somatomedin activity had been dissociated, the half life of this recombined somatomedin activity was prolonged to approximately 2 h. These experiments suggested that (1) the half life of somatomedin



and 0.5 ml of Ca^{2+} - Mg^{2+} -free phosphate-buffered saline (Dulbecco's PBS) for 3 h at 30 °C. The incubation mixture was then applied to a 180-ml Sephadex G-200 column (2.6 × 40 cm) equilibrated and eluted with Dulbecco's PBS at 4 °C. Fractions of 2.2 ml were collected. Both ^{125}I -MSA radioactivity (●) and protein content (A_{280}) are plotted against fraction number and percentage of total bed volume. The excluded volume (V_0) was marked with blue dextran, and the included volume (indicated by the arrow) was marked with dinitrophenylalanine. The main protein peaks (---) from left to right correspond to macroglobulins, gammaglobulins, and albumin. *a*, Elution pattern of normal rat serum incubated with ^{125}I -MSA. Peaks of ^{125}I -MSA are denoted by Roman numerals in order of decreasing molecular size. *b*, Elution pattern of serum obtained from Hx male Sprague-Dawley rats at least 10 d after hypophysectomy (performed when they were 90–100 g body weight)⁸. *c*, Elution pattern of serum obtained from Hx rats after 10 d of twice daily intraperitoneal injections of 100 µg of bovine growth hormone (chronic GH)⁸. *d*, Elution pattern of serum obtained from Hx rats after intraperitoneal injection of 100 µg of bovine growth hormone every 8 h for 24 h (acute GH)⁸.

activity in the circulation was determined by its binding to large serum proteins and (2) these binding proteins were regulated by growth hormone.

To investigate directly the binding of a somatomedin-like polypeptide to rat serum, we have used multiplication stimulating activity (MSA), a 10,000-da'ton polypeptide purified from the conditioned media of a line of normal rat liver cells in culture^{9,10}. MSA has physicochemical similarities to the somatomedins purified from human plasma and shares with these polypeptides the ability to interact with the same receptors in various tissues and to stimulate

growth-related processes of certain cells and tissues in culture^{2,9–13}. Specifically, the MSA preparation used for the studies reported here competes for somatomedin A binding to placental membranes¹². Conversely, somatomedin A competes for the binding of this MSA to chick embryo fibroblasts, human fibroblasts and rat liver membranes¹². An MSA preparation of similar specific activity also stimulates sulphate incorporation into chick embryo sternal cartilage at a concentration of 25 ng ml⁻¹ (unpublished experiments of K. Gibson and E. R. Froesch) and competes for NSILA-s binding to chick embryo fibro-

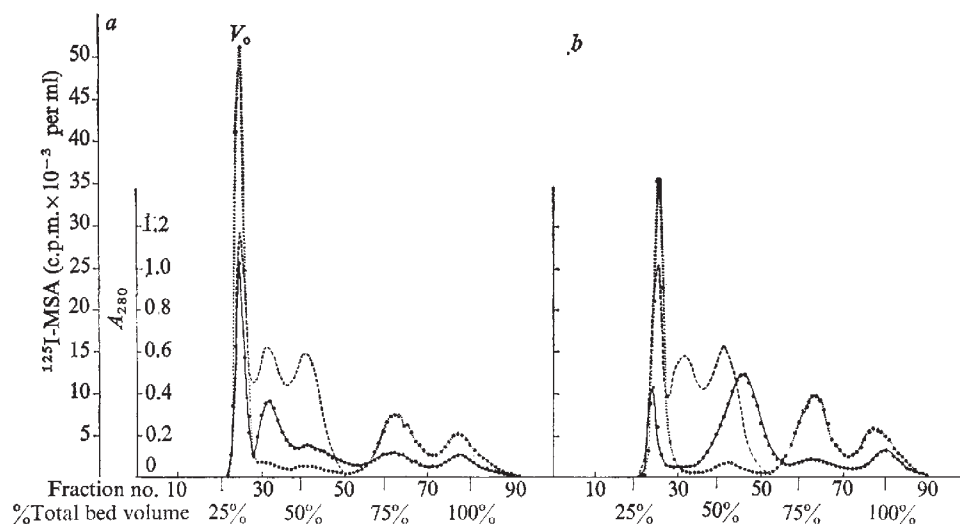


Fig. 2 Competition for ^{125}I -MSA binding to rat serum by unlabelled MSA. Normal rat serum (*a*) or Hx rat serum (*b*) were incubated for 3 h at 30 °C with 6 ng of ^{125}I -MSA in the presence (○--○) or absence (●—●) of 10 µg of unlabelled MSA as in Fig. 1. The samples were applied to a column of Sephadex G-200 and analysed as in Fig. 1. Protein content (---) and radioactivity of the eluted fractions are plotted.

blasts (unpublished experiments of J. Zapf and E. R. Froesch).

^{125}I -MSA was incubated with normal rat serum and chromatographed on Sephadex G-200. Radioactivity appeared in five peaks, I-V (Fig. 1a). Only the binding to peaks II and III was specific, that is, it was abolished in the presence of excess ($10\text{ }\mu\text{g ml}^{-1}$) unlabelled MSA (Fig. 2). Binding of ^{125}I -MSA to peak I was nonspecific (Fig. 2), although the possibility of some specific binding of MSA to proteins in the peak I region has not been excluded completely. Peak IV represented free ^{125}I -MSA and peak V represented free ^{125}I as determined by the elution pattern of carrier free Na^{125}I .

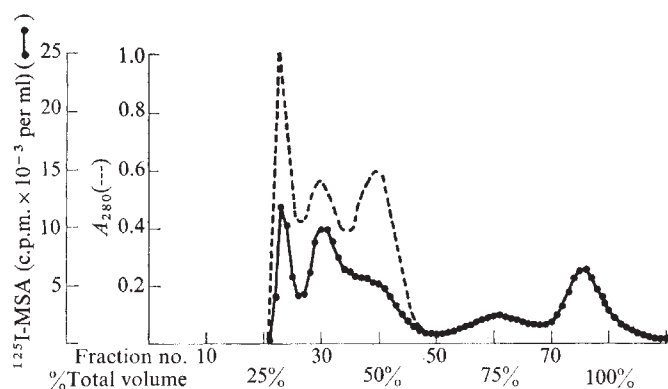


Fig. 3 MSA binding to a mixture of equal volumes of normal and Hx rat serum. ^{125}I -MSA (6 ng) was incubated with 0.25 ml of Hx rat serum, 0.25 ml of normal rat serum and 0.5 ml of Dulbecco's PBS for 3 h at 30°C . The incubation mixture was then chromatographed on the same column of Sephadex G-200 as in Figs 1 and 2. Fractions were analysed for ^{125}I (●) and protein (---).

The pattern of MSA binding to normal and Hx rat serum differed strikingly. Peak II, which represented the major specific binding area for MSA in normal rat serum, was absent in Hx rat serum. In this serum, ^{125}I -MSA bound specifically only to the peak III region (Figs 1b and 2b). MSA also bound to peak III in normal rat serum but to a lesser extent than in Hx rat serum (Fig. 1a and b).

When peaks II and III that had been isolated respectively from chromatography on Sephadex G-200 of normal and Hx rat serum were rerun individually over the same column, they eluted in a position identical to their position in whole serum (data not shown). This experiment excluded the conversion of one peak to the other. Furthermore, on the basis of the chromatographic pattern of a mixture of equal volumes of normal and Hx rat serum that had been incubated with ^{125}I -MSA, Hx rat serum did not inhibit binding of peak II (Fig. 3). Thus, an inhibitory component in Hx rat serum could not account for the absence of peak II MSA in Hx serum. These results indicated that MSA binds to distinct species, presumably proteins, in normal and Hx sera.

The different profiles of MSA binding to normal and Hx rat serum suggested that the binding pattern depended on growth hormone. This was confirmed by examining sera from Hx rats treated with growth hormone for different periods (Fig. 1c and d). Peak II, absent from Hx rat serum, reappeared after only 24 h of growth hormone treatment of Hx rats (Fig. 1d). After 10 d of growth hormone treatment, there was a further increase in the binding of ^{125}I -MSA to peak II that resulted in a binding profile virtually identical to that obtained with normal rat serum (Fig. 1c). Thus, although growth hormone increases the level of peak II MSA binding in Hx rats, the mechanism by which growth hormone enhances peak II binding remains undefined.

Growth hormone could induce the synthesis or release of the peak II-binding protein or it could modify a pre-existing protein such as peak III. Together with the observations of Cohen and Nissley⁸, the above experiments suggest that the peak II binding protein prolongs the half life of somatomedin activity in serum. That is, serum containing the peak II MSA-binding protein yields a longer half life of somatomedin activity when injected into Hx animals than does serum without this binding protein.

The difference between MSA binding in normal and Hx rat serum was investigated further by a competitive binding assay (Fig. 4). After incubation of ^{125}I -MSA with unfractionated rat serum, unbound ^{125}I -MSA was separated from ^{125}I -MSA bound to serum proteins by adsorption to activated charcoal⁹. The binding of MSA to serum proteins was highly specific. Unlabelled MSA inhibited ^{125}I -MSA-binding to normal rat serum. Neither MSA that had been biologically inactivated by reduction and alkylation, human growth hormone, glucagon, human placental lactogen nor insulin at high concentrations competed effectively for ^{125}I -MSA binding (data not shown).

The specific binding of ^{125}I -MSA to normal rat serum was more than twice that to Hx rat serum (Fig. 4). In addition, serum from Hx rats treated with growth hormone for 10 d

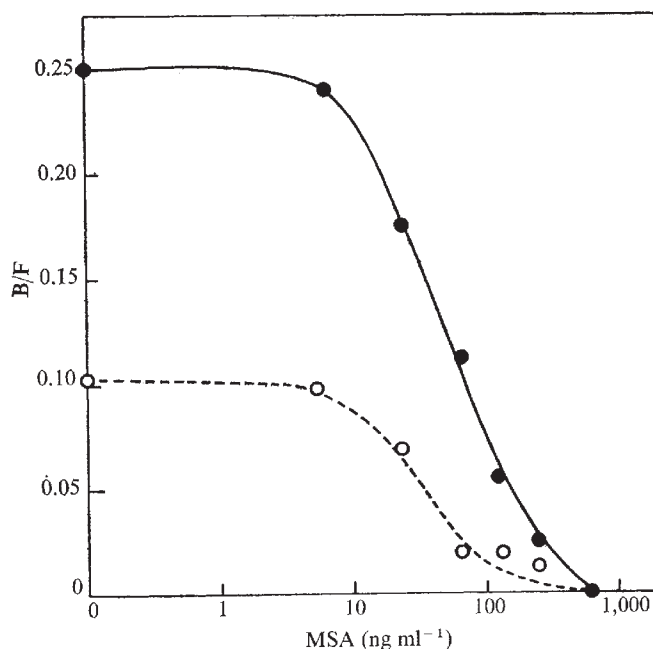


Fig. 4 Whole serum competitive binding assay. Normal (●) and Hx (○) rat sera were dialysed for 12 h at 4°C against 250 volumes of Dulbecco's PBS and diluted with 3 volumes of the same buffer. Dialysed, diluted serum (25 μl) was incubated with 200 pg of ^{125}I -MSA in Dulbecco's PBS containing 0.2% human serum albumin in the presence or absence of unlabelled MSA at 30°C in a total of 0.4 ml. After 3 h of incubation, 0.5 ml of Dulbecco's PBS containing activated charcoal in 2% human serum albumin was added to the reaction mixture⁹. The samples were equilibrated for 30 min at 0°C and centrifuged at 2,500g for 10 min. The ^{125}I radioactivity in both supernatant liquid and the charcoal pellet were determined. (In a separate experiment, free ^{125}I -MSA quantitatively adsorbed to the activated charcoal. ^{125}I -MSA bound to serum proteins was not adsorbed.) The ^{125}I -MSA bound to serum proteins in the presence of unlabelled MSA ($1\text{ }\mu\text{g ml}^{-1}$) (nonspecific binding) was equal in both normal and Hx serum and has been subtracted from the total MSA before calculating the bound MSA to free MSA ratio (B/F). Nonspecific binding, equalled 24% of total binding for this experiment. B/F is plotted against increasing log concentrations of unlabelled MSA added to the reaction mixture. The binding of MSA to normal and growth hormone-treated Hx serum would underestimate specific MSA binding to these sera if endogenous somatomedins compete with ^{125}I -MSA for the binding protein(s).

bound 1.5 times the ^{125}I -MSA bound by serum from untreated Hx rats (data not shown). The concentration of unlabelled MSA required to displace 50% of the specifically bound ^{125}I -MSA was approximately the same in normal, Hx and growth hormone-treated Hx serum (Fig. 4). These data suggest that normal and Hx rat serum have similar binding affinities but different binding capacities. This interpretation is supported by the predominant binding of MSA to peak II in the mixing experiment (Fig. 3). We presume that the difference in specific MSA binding by unfractionated normal and Hx serum reflects differences in the levels of peak II and peak III-specific binding proteins in these sera.

In summary, we have shown by Sephadex chromatography and a competitive binding assay that normal rat serum specifically binds a somatomedin-like polypeptide; that this binding (peak II) is growth hormone dependent, and that its presence correlates with a prolonged half life of circulating somatomedin activity. The half life of somatomedin activity in Hx rat serum is short in spite of the presence of a specific somatomedin-binding protein (peak III). This could be explained by reduced binding capacity in Hx serum (Fig. 4) or by differences in the clearance or degradation of peak III or its complex with somatomedin.

Whatever the mechanism by which growth hormone alters somatomedin binding in serum, the dependence of somatomedin binding and half life on growth hormone adds an important 'fine-tuning' modulation to the control of growth by growth hormone. Indeed, the levels of a somatomedin-binding protein may determine the levels of somatomedin in the circulation. The absence of the peak II somatomedin-binding protein in Hx rats suggests that it may be difficult to produce *in vivo* growth-promoting effects with purified somatomedins due to the predicted rapid disappearance of these somatomedins from the circulation of the recipient Hx animal. Confirmation of this hypothesis awaits the purification of the somatomedin-binding protein and its injection into Hx animals in combination with somatomedin.

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Serum can initiate DNA synthesis in cells rendered unresponsive to insulin and somatomedin

AN understanding of growth control in animal cells requires an appreciation not only of cellular events important to multiplication, but also of exogenous agents which either initiate or influence the 'decisions' of a cell to reproduce. Serum has been reported to contain factors required for cell movement¹, viability², ion transport³ and control of cell density⁴. In addition, peptides from tissue or plasma have been identified (nerve growth factor (NGF)⁵, somatomedin⁶, non-suppressible insulin-like activity (NSILA)⁷, multiplication-stimulating activity (MSA)⁸, epidermal growth factor⁹, fibroblast growth factor¹⁰) which seem to have a physiological role in the initiation of cell proliferation. Somatomedin, NSILA and MSA are probably similar, if not identical, substances and, like NGF, seem to be structurally related to insulin¹¹⁻¹³. The ability of insulin to initiate DNA synthesis in various cells¹⁴⁻¹⁷ may be due to cross reactivity of the hormone with receptor sites concerned with cell multiplication¹². Recent reports suggest that serum contains additional substances that can influence the ability of a cell to respond to agents which initiate DNA synthesis^{10,18,19}. While investigating the regulatory roles of insulin and serum in the initiation of DNA synthesis in quiescent chick embryo fibroblasts (CEF), we have found a distinctive difference in the initiation capacity of these agents after the inhibition of protein synthesis. We present here evidence for the existence of a regulatory component(s) in serum which recruits cells for growth from a stage in the cell cycle position (G_0 ?) which is refractory to mitogenic peptides.

As shown before²⁰⁻²², quiescent CEF initiate DNA synthesis 6-8 h after exposure to insulin or serum. Based on the incorporation of ^3H -thymidine into trichloroacetic acid (TCA)-insoluble material, the highest levels of DNA synthesis were initiated by insulin at a concentration of 1-5 $\mu\text{g ml}^{-1}$ and approximated only 20-50% of the maximum levels stimulated by 3% serum (Fig. 1 legend). When serum-deprived CEF were treated with cycloheximide (5 $\mu\text{g ml}^{-1}$) before exposure to insulin, however, the subsequent insulin-induced DNA synthesis in washed, inhibitor-free cultures was found to be significantly reduced in comparison with previously uninhibited insulin-stimulated cultures. Figure 1 shows that the drug-induced inhibition of insulin-stimulated DNA synthesis was time dependent. Exposure to cycloheximide for 6-8 h was required to reduce ^3H -thymidine incorporation to 30-50% of that seen in uninhibited, hormone-stimulated cultures (Fig. 1). The percentage of insulin-treated cells with labelled nuclei was similarly decreased from 16% in uninhibited cells to 3% in inhibitor-free cells previously exposed to cycloheximide for 8 h (Fig. 2). In serum-deprived cultures, cycloheximide (5 $\mu\text{g ml}^{-1}$) inhibited incorporation of ^3H -amino acids into TCA-insoluble material by 70-80% as previously shown²⁴. Immediately after the cells had been washed free of inhibitor, the incorporation of labelled amino acids returned to levels characteristic of serum-deprived control cultures (Fig. 1, inset). In contrast to the inhibition of insulin-stimulated DNA synthesis after exposure to cycloheximide, a similar cycloheximide treatment failed to reduce the serum-stimulated response (Figs 1 and 2). Similar results have been obtained with four different lots of calf serum. The fully recoverable serum-stimulated response has also been demonstrated with serum dialysed at a neutral pH (data not shown).

By measuring the DNA content of cultures, these results were further substantiated and extended to somatomedin, also an initiator of DNA synthesis in quiescent CEF¹². In

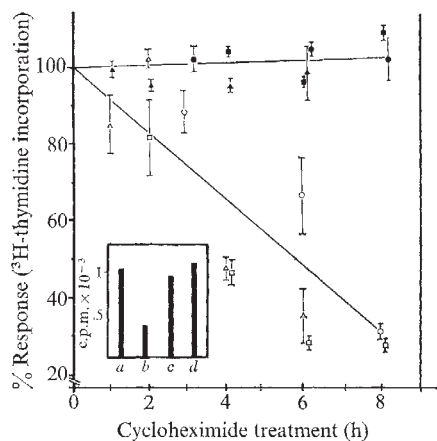


Fig. 1 Effect of cycloheximide pretreatment on insulin or serum-stimulated incorporation of ^3H -thymidine into TCA-insoluble material. CEF were prepared by trypsinisation of 11-d-old embryos²³. Wells (diameter 16 mm) containing 12-mm glass coverslips were each seeded with 9×10^5 cells in 3% calf serum minimal essential medium (MEM) (1 ml per well). After incubation for 72 h (37 °C) in a humidified 5% CO_2 atmosphere, confluent cells (5×10^4 – 8×10^4 cells cm^{-2}) were washed once with Hanks' balanced salt solution (BSS) and further incubated in 1 ml of serum-free MEM for 24 h. Cells were then treated with cycloheximide at a final concentration of $5 \mu\text{g ml}^{-1}$ by the addition of small 10- μl samples from a concentrated stock of the drug. After a maximum cycloheximide treatment period of 8 h, all cells were washed twice with BSS, and then MEM with insulin ($5 \mu\text{g ml}^{-1}$) or 3% calf serum was added to wells in 1-ml volumes. After 6 h, 0.4 μCi of ^3H -thymidine (^3H -methyl, specific activity 64 Ci mmol^{-1} , Nuclear Dynamics) in MEM was added to each well. Twenty-four hours after the addition of hormone or serum, cells were washed once with cold saline (0.85%) and TCA-soluble material was extracted with cold 5% TCA (1 ml per well). After 10 min, the TCA was discarded and fresh 5% TCA added for a further 5 min. Coverslips were dipped in 95% ethanol and dried in counting vials at 50 °C. The TCA-insoluble radioactivity (c.p.m. per coverslip) was determined after the addition of Omnifluor-toluene scintillation fluid (Δ , $\blacktriangle$; $\square$, $\blacksquare$). In one experiment, ($\circ$, $\bullet$), after the second TCA wash coverslips were placed in small glass vials and the cells were digested with Protosol (New England Nuclear) for 2 h at 50 °C before addition of scintillation fluid. Percentage response is reported as the c.p.m. stimulated by insulin (Δ , $\square$, $\circ$) or serum ($\blacktriangle$, $\blacksquare$, $\bullet$) in three experiments after cycloheximide treatment for 1–8 h over uninhibited insulin-stimulated c.p.m. (Δ , 25,820 c.p.m. $\pm$ 61; $\square$, 13,360 c.p.m. $\pm$ 960; $\circ$, 39,095 c.p.m. $\pm$ 4174) or serum-stimulated c.p.m. ($\blacktriangle$, 54,414 c.p.m. $\pm$ 1537; $\blacksquare$, 50,160 c.p.m. $\pm$ 2606; $\bullet$, 84,662 c.p.m. $\pm$ 602), respectively. ^3H -thymidine incorporation in control cultures (MEM stimulated) for the three experiments averaged 2,545 c.p.m. The range and mean of duplicate determinations are shown. Inset, ^3H -amino acid incorporation into TCA-insoluble material in inhibited and uninhibited cultures. After treatment with (b, c) or without (a, d) cycloheximide for 6 h, cultures a and b were pulse labelled for 30 min at 37 °C with 2.5 μCi of ^3H -amino acids (Schwartz Mann, protein hydrolysate, 1 mCi ml^{-1}). c and d were washed twice with BSS and immediately pulsed with ^3H -labelled amino acids in fresh serum-free MEM for 30 min. Coverslips were then washed four times with cold saline and processed and counted as described above. The variation from the mean of duplicate samples was less than 10% in all cases. Amino acid incorporation was inhibited 71% in the presence of cycloheximide.

uninhibited cultures the DNA content 24 h after exposure to insulin, somatomedin (0.2 U ml^{-1}) or serum increased 18%, 20% and 50%, respectively (Fig. 3). In cells previously inhibited with cycloheximide (+CH) for 8 h, the increases in DNA initiated by insulin and somatomedin were either not observed or considerably reduced, again in contrast to the serum-initiated increases in DNA (Fig. 3). These data argue against the possibility that cycloheximide dissociates ^3H -thymidine transport and DNA synthesis, as shown with cytochalasin B treatment of 3T3 cells²⁰.

To explain the failure of the drug to diminish serum-stimulated DNA synthesis, it could be argued that the hormone-responsive cells inhibited by cycloheximide are

not part of the larger population of cells that responds to serum. Incorporation of TCA-insoluble ^3H -thymidine and autoradiography show however, that non-additive levels of DNA synthesis are obtained in cultures exposed to either of the hormones plus the maximum stimulatory concentration of serum (data not shown). This suggests that each of the hormones stimulates DNA synthesis in cell populations that also initiate DNA synthesis in response to serum. Similar findings have been reported before⁷.

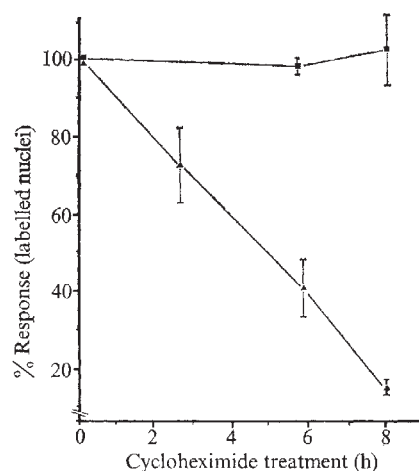


Fig. 2 Effect of cycloheximide pretreatment on insulin or serum-stimulated labelled nuclei. CEF were prepared as in Fig. 1. Wells (35 mm diameter) containing 22 $\times$ 22-mm glass coverslips were each seeded with 3×10^6 cells in 3% calf serum MEM (3 ml per well). After incubation for 72 h (37 °C) in a humidified 5% CO_2 atmosphere, confluent cells were washed once with BSS and further incubated in 2.5 ml of serum-free MEM. After 24 h in serum-free MEM, cells were treated with cycloheximide at a final concentration of $5 \mu\text{g ml}^{-1}$ by addition of 25- μl samples from a concentrated stock of the drug. After a maximum cycloheximide treatment period of 8 h, cells were washed twice with BSS, and then MEM with $5 \mu\text{g ml}^{-1}$ insulin or 3% calf serum was added to wells in 2.5-ml volumes. After 6 h 1 μCi of ^3H -thymidine in MEM was added to each well. Twenty-four hours after the addition of hormone or serum, cells were washed once with saline and processed for autoradiography as before²⁰. Percentage response is reported as percentage of insulin- (Δ) or serum- ($\blacksquare$) stimulated cells with labelled nuclei after cycloheximide treatment for 3, 6 or 8 h over the percentage of uninhibited insulin-stimulated cells with labelled nuclei ($16\% \pm 3$) or serum-stimulated cells with labelled nuclei ($58\% \pm 6$), respectively. The percentage of cells with labelled nuclei after addition of MEM alone was 3%. The range and mean of duplicate determinations are shown.

The kinetics of the fully recoverable serum-stimulated DNA synthesis in CEF after cycloheximide treatment was monitored by pulse labelling cells with ^3H -thymidine at various times after addition of serum. Figure 4 shows that compared with uninhibited control cultures, both the onset and peak of serum-stimulated DNA synthesis were delayed by 3–5 h in cycloheximide-treated cells. In our hands, the timing of DNA synthesis in uninhibited insulin-stimulated CEF is similar to that of uninhibited serum-stimulated cells^{20,27}.

The delayed onset of DNA synthesis in inhibited cells after exposure to serum could account for the inability of the hormones to stimulate DNA synthesis, for during the additional time needed by cells to regain the capacity to synthesise DNA, the hormones could have been degraded. This seems unlikely, however, because media containing insulin $5 \mu\text{g ml}^{-1}$ remained fully active in stimulating DNA synthesis for at least 16 h after incubation with inhibited or uninhibited cultures. This latter observation (data not shown) was made by measuring ^3H -thymidine incorporation into TCA-insoluble material stimulated in fresh cell monolayers exposed to the 'used' insulin-containing media.

Thus, the ability of inhibited cells to synthesise DNA after

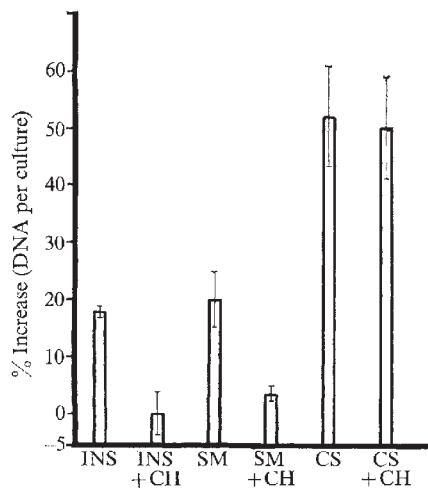
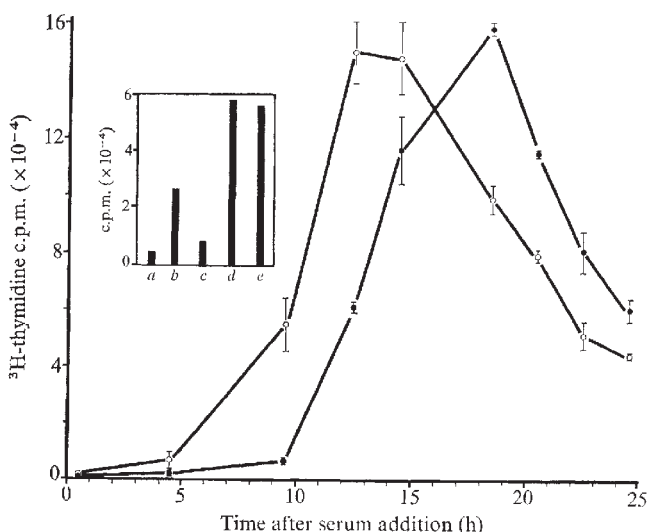


Fig. 3 Effect of cycloheximide pretreatment on the DNA content of CEF after the addition of insulin (INS), somatomedin (SM) or calf serum (CS). CEF were prepared as for Fig. 1. Plastic flasks (72 cm²) were each seeded with 2.2×10^7 cells in 3% calf serum MEM (15 ml per flask). After incubation for 72 h (37 °C) in a humidified 5% CO₂ atmosphere, confluent cells were washed once with BSS and further incubated in 15 ml of serum-free MEM. After 24 h in serum-free MEM, cultures were treated with cycloheximide (5 µg ml⁻¹) and after 8 h were washed twice with 15 ml of BSS. Cells were exposed to MEM, INS (5 µg ml⁻¹), SM (0.2 µg ml⁻¹) or 3% CS. Twenty-four hours later the cells were washed once with saline, trypsinised (0.5% trypsin), pelleted by centrifugation and counted. The DNA content per flask (3×10^6 – 5×10^6 cells) was determined after treatment of cells with 1 N perchloric acid at 70 °C for 20 min, using the method of Burton²⁵. The percentage increase in DNA content was determined using the A_{600} values obtained for MEM-stimulated cells as baseline (0% increase). For each bar, the variation and mean of two experiments is given. In each experiment, all points were determined in duplicate and averaged.

exposure to serum seems to require additional time-dependent cellular changes not required of uninhibited cells. In view of the inability of insulin or somatomedin to induce DNA synthesis in cycloheximide-treated cells, these further changes seem to be initiated by a serum component(s) distinct from those which signal DNA synthesis directly. In this regard, serum seems to be performing a more complex function than simply supplying inhibited cells with an exogenous nutrient needed for DNA synthesis. If this were the case, the timing of DNA synthesis might be expected to be unaffected by cycloheximide treatment²⁸.



To summarise and further clarify our ideas, we suggest that the inhibition of protein synthesis results in the loss of some cellular protein(s) required directly or indirectly for the induction of DNA synthesis by insulin and somatomedin, and that these agents cannot reinitiate the synthesis of this protein. An additional regulatory component(s) in serum is proposed that can signal the synthesis of this missing cellular protein(s), thus permitting the subsequent induction of DNA synthesis by factors in serum functionally analogous to insulin and somatomedin. In terms of the eukaryotic cell cycle, this additional serum component(s) could have a role in the recruitment of cells from G₀ into G₁.

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Fig. 4 Effect of cycloheximide pretreatment on the timing of DNA synthesis in CEF after the addition of serum. CEF were prepared as for Fig. 1. Glass bottles (11 cm²) were each seeded with 4.5×10^6 cells in 3% calf serum MEM (3 ml per bottle). After incubation for 72 h in a humidified 5% CO₂ atmosphere, confluent cells were washed once with BSS and further incubated in 3 ml of serum-free MEM. After 24 h in serum-free MEM, cultures were treated with or without cycloheximide (5 µg ml⁻¹) and after 8 h were washed twice with 3 ml of BSS. Cells were treated with MEM containing 3% calf serum and pulse labelled with ³H-thymidine (5 µCi per bottle) for 1-h intervals at the times indicated. Pulse-labelled cells, washed once with cold saline (3ml) were scraped into 1 ml of saline to which 7.5% TCA was added. TCA-insoluble material was collected by centrifugation, washed once with 5% TCA, and dissolved in 0.1 M NaOH (0.75 ml). Samples of 0.25 ml were added to 5 ml of scintillation fluid (Triton X-Omnifluor-toluene) and counted. The range and mean of duplicate determinations are shown. ○, Uninhibited cultures; ●, cycloheximide-pretreated cultures. Inset, DNA synthesis in control cultures for 24 h after the addition of hormone or serum. After treatment with (c, d) or without (a, b, e) cycloheximide for 8 h, cells were washed and exposed to a, MEM; b, c, insulin; d, e, serum as described above. ³H-thymidine (0.4 µCi per bottle) was added 6 h after the addition of hormone or serum. Cells were collected 18 h later and the TCA-insoluble material was prepared and counted as described above. Insulin-stimulated DNA synthesis was inhibited 69% by cycloheximide pretreatment.

Magnesium required for serum-stimulation of growth in cultures of chick embryo fibroblasts

THE addition to culture medium of phosphorylated compounds which preferentially bind Mg^{2+} substantially reduces the rate of DNA synthesis and metabolism of chick embryo fibroblasts (CEF)¹. This has suggested that Mg^{2+} might be an intracellular regulator for coordinate control of the rate of cell growth and metabolism in animal cells¹. We report here that the omission of Mg^{2+} from the culture medium can have considerable effects on cell growth and macromolecular synthesis by CEF if the Mg^{2+} is withdrawn at the time cells are being stimulated to grow by means of an increase in the concentration of serum in the medium. These observations are discussed in terms of a model in which the intracellular concentration of free Mg^{2+} coordinately regulates the rate of metabolism and cell growth through its role as an essential cofactor for transphosphorylation reactions.

Primary cultures of CEF were prepared as before². Secondary cultures were plated 3–5 d before use at 10^6 cells per 60-mm Falcon culture dish in medium 199, containing 2% tryptose phosphate broth (TPB) and 1% chicken serum. During experimental manipulation, medium 199 containing 2% TPB was again used at the indicated Mg^{2+} concentration, but with dialysed serum present. Experimental techniques for isotopic labelling, scintillation counting and autoradiography have been described before^{3,4}. Concentrations of Mg^{2+} in the culture medium were determined with a Perkin-Elmer atomic absorption spectrophotometer.

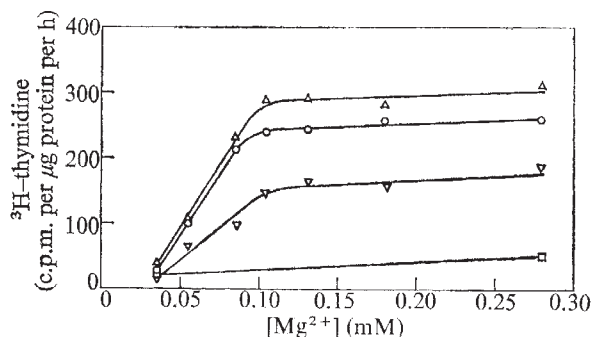


Fig. 1 3H -thymidine incorporation by CEF as function of the concentration of Mg^{2+} and calf serum. Four-day-old cultures were changed to fresh medium at the indicated concentrations of Mg^{2+} and calf serum; 20 h later the rate of DNA synthesis was measured by 3H -thymidine ($0.6 \mu Ci ml^{-1}$ medium) incorporation into acid insoluble material for 60 min (calf serum: Δ , 8%; $\circ$, 4%; ∇ , 1%; $\square$, 0.0%).

Stimulation of cell growth by serum causes an increase in the rate of 3H -thymidine incorporation by CEF that is proportional to the increase of the number of cells in the S phase of the cell cycle⁵. But as Fig. 1 shows, reducing the Mg^{2+} concentration of the medium from the standard 0.8 mM concentration to 0.04 mM prevents the increase of 3H -thymidine incorporation. Below 0.1 mM Mg^{2+} the rate of 3H -thymidine incorporation was directly proportional to the Mg^{2+} concentration of the medium at any concentration of calf serum (or chicken serum) from 1% to 8%.

There have been reports that the addition of Mg^{2+} to culture medium is not essential for the growth of mouse L cells⁶ and HeLa cells⁷. We suppose that either of those cell lines have a slightly lower Mg^{2+} requirement for growth or, in the case of the report on mouse L cells⁶, the medium

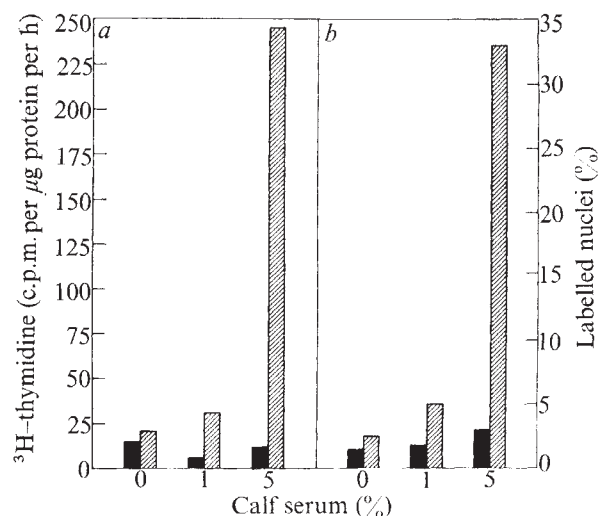


Fig. 2 Comparison of total 3H -thymidine incorporation and autoradiography as measures of the rate of DNA synthesis. Four-day-old cultures were changed to medium containing 0.04 mM Mg^{2+} and the indicated concentrations of dialysed calf serum. After 20 h, half of the cultures were stimulated to grow by increasing the Mg^{2+} concentration of the media to 0.84 mM. Twenty hours later 3H -thymidine ($0.6 \mu Ci ml^{-1}$) was added to the medium for 60 min. *a*, Half of the cultures were used to determine total 3H -thymidine incorporation into acid insoluble material; *b*, parallel cultures prepared for autoradiography as described before⁴. Solid columns, 0.04 mM Mg^{2+} ; hatched columns, 0.84 mM Mg^{2+} .

used might have contained more Mg^{2+} as a contaminant than was present in ours. As both of these cell lines have malignant characteristics, a reduced requirement for Mg^{2+} could also be characteristic of malignant transformation.

Figure 2 shows that Mg^{2+} deprivation of cells reduced 3H -thymidine incorporation mainly by decreasing the rate

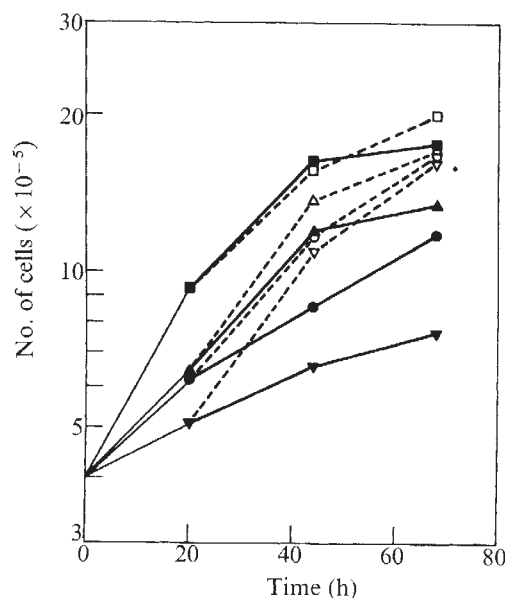


Fig. 3 Reversibility of inhibition of cell growth by Mg^{2+} deprivation. At zero time 5×10^5 CEF in medium 199 ($-Mg^{2+}$) containing 2% TPB, 5% dialysed calf serum and the indicated concentrations of Mg^{2+} were added to 60-mm culture plates. In these conditions, approximately 80% of the cells attach to the plates by 3 h. At 20 h half of all of the cultures were changed to medium containing the standard 0.84 mM Mg^{2+} . Then at 20, 44 and 68 h the number of cells on each plate was determined, after trypsin treatment, with a Coulter counter. Original medium: ∇ , 0.04 mM Mg^{2+} ; $\bullet$, 0.06 mM Mg^{2+} ; Δ , 0.11 mM Mg^{2+} ; $\blacksquare$, 0.84 mM Mg^{2+} . The open symbols with dashed lines indicate the corresponding cultures changed at 20 h to medium containing 0.84 mM Mg^{2+} .

of entry of cells into the S phase of the cell cycle, not just by slowing the rate of DNA synthesis in individual cells. When Mg^{2+} was added back to the culture medium of cells that had been deprived of Mg^{2+} for 24 h, there was stimulation of both 3H -thymidine incorporation and percentage 3H -thymidine-labelled nuclei: this was directly related to the serum concentration in which the cells had been maintained during this period. In the case of Mg^{2+} -deprived cells maintained in medium containing 5% calf serum there was a 17-fold increase in the rate of 3H -thymidine incorporation 20 h after addition of Mg^{2+} to the culture medium. Autoradiograms of parallel cultures revealed a 10-fold increase in the number of 3H -thymidine-labelled nuclei at this time. If 3H -thymidine incorporation accurately reflected the rate of DNA synthesis, then after the addition of Mg^{2+} individual cells made DNA only 1.7 times faster, but 10 times more cells were in S phase. Likewise with Mg^{2+} -deprived cultures maintained in 1% calf serum there was a fivefold increase in 3H -thymidine incorporation and a threefold increase in the percentage of labelled nuclei 20 h after addition of Mg^{2+} .

Figure 3 shows the inhibition of cell multiplication by Mg^{2+} deprivation, and its reversal when Mg^{2+} was restored. At zero time 5×10^5 CEF in medium containing the indicated concentrations of Mg^{2+} were plated on 60-mm culture dishes. About 80% of the cells became attached at all Mg^{2+} concentrations. At 20 h half of all the cultures were moved to medium containing 0.84 mM Mg^{2+} . Then at 20, 44 and 68 h the cells on each plate were counted, after trypsin treatment, with a Coulter counter. By 20 h after plating-out of cultures, the cells growing in medium containing 0.84 mM Mg^{2+} had doubled in number and continued

to multiply until 48 h. In cultures with 0.11 mM Mg^{2+} or less in the growth medium, however, the growth rate showed a dependence on Mg^{2+} concentration. Mg^{2+} deprivation did not reduce the growth rate of these cells by simply decreasing their viability. Addition of 0.8 mM Mg^{2+} to the growth medium restored the increase of these cells to the maximum rate and allowed them to achieve the population density of cells in control cultures. This illustrates the possible potential of alterations in intracellular Mg^{2+} for the physiological regulation of cell growth.

As well as preventing serum-stimulation of cell growth, Mg^{2+} deprivation resulted in substantial decreases in the rates of synthesis of RNA and protein (Fig. 4). Twenty hours after the Mg^{2+} concentration had been decreased to 0.04 mM the rates of incorporation of 3H -proline and 3H -uridine were reduced 3.5-fold and 10-fold, respectively, from the level of control cells growing in 5% calf serum. Therefore Mg^{2+} deprivation seems not only to inhibit the rate of growth of CEF but also to inhibit their rate of macromolecular synthesis and presumably metabolism as a whole. This coordinate metabolic response of cells when the extracellular concentration of Mg^{2+} is reduced is also displayed on removal of serum or contact inhibition of growth⁴. Many agents that stimulate growth, such as serum, insulin and pH manipulation of the medium, seem to increase cellular functions in a coordinate manner⁸. We believe that the intracellular concentration of free Mg^{2+} has a central role in this coordinate metabolic response of animal cells to all these agents. Other divalent cations including Zn^{2+} (ref. 9) and Ca^{2+} (ref. 10) may have subsidiary roles.

In previous experiments, in which $Na_2P_2O_7$ or adenine nucleotides have been used to complex Mg^{2+} in the medium, similar effects on cellular metabolism have been noted and a model for coordinate control of cell metabolism has been proposed¹. According to this model, intracellular Mg^{2+} is limiting in the formation of adenine nucleotide- Mg^{2+} complexes, essential substrates for transphosphorylation reactions. Transphosphorylation reactions, in turn, are viewed as the regulatory steps in metabolic pathways. This seems to be true in the glycolytic pathway, the one example where all the substrates and products of the pathway are readily assayed, since a selective activation of transphosphorylation reactions takes place when rates of glycolysis are increased^{11,12}. The control of the intracellular level of free Mg^{2+} is brought about, in the context of this model, by changes in the competitive binding capacities of membranes and adenine nucleotides for Mg^{2+} . Because most of the cellular Mg^{2+} are presumably not free inside the cell but bound to membranes and other cellular constituents^{13,14}, serum and other agents that stimulate cell growth are postulated to alter membrane structure so as to release small amounts of membrane-bound Mg^{2+} . Chelation of Ca^{2+} in the culture medium by EGTA has been reported to inhibit cell growth in chick fibroblasts¹⁰: this might be the result of a competitive binding of Mg^{2+} and Ca^{2+} to membranes. The removal of sufficient Ca^{2+} from cellular membranes could result in a replacement by Mg^{2+} (ref. 13), resulting in a lower intracellular concentration of free Mg^{2+} . Indeed, it has been shown that the removal of Ca^{2+} from the medium markedly increases the sensitivity of cells to Mg^{2+} deprivation¹⁵.

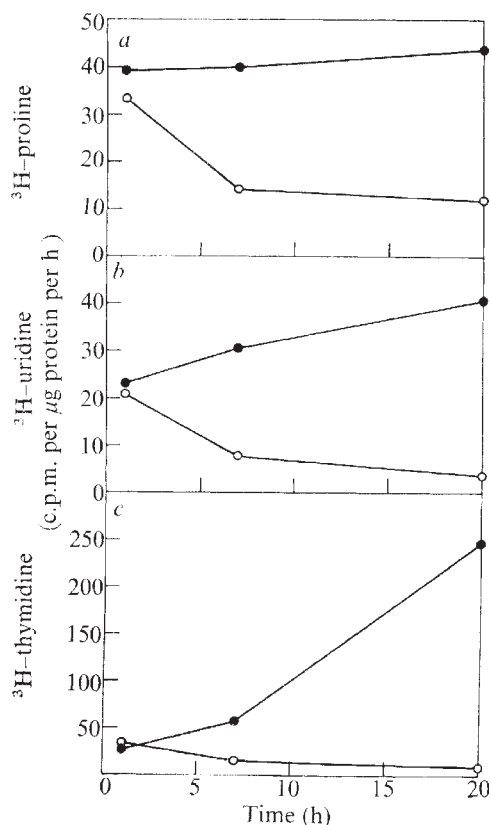
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Fig. 4 Effect of Mg^{2+} deprivation on 3H -proline, 3H -uridine and 3H -thymidine incorporation. Five-day-old cultures were changed to medium containing 5% dialysed calf serum and either 0.84 mM Mg^{2+} or 0.04 mM Mg^{2+} . At various times the rates of synthesis of protein, RNA and DNA were determined by incorporation of: *a*, 3H -proline (1.5 $\mu Ci ml^{-1}$); *b*, 3H -uridine (0.1 $\mu Ci ml^{-1}$); or *c*, 3H -thymidine (0.6 $\mu Ci ml^{-1}$), respectively, into acid-insoluble material during 60-min incubations. $\circ$, 0.04 mM Mg^{2+} ; $\bullet$, 0.84 mM Mg^{2+} .



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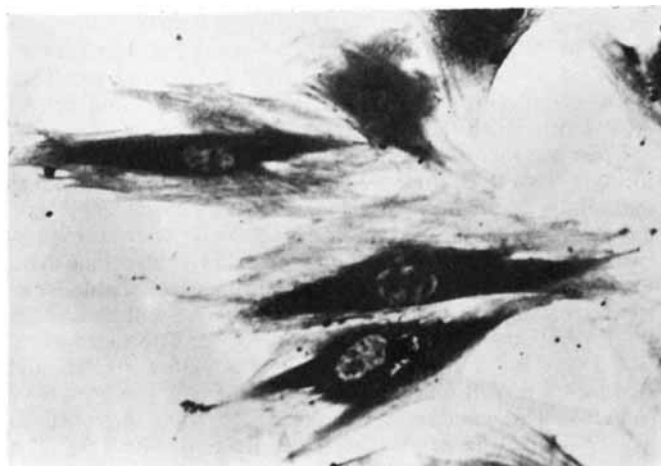
C1q production and secretion by fibroblasts

THE serum protein C1q is a subcomponent of the first component of the complement system. Combination of C1q with antigen-antibody complexes activates the other sub-components of C1 (C1r and C1s) and sets in motion a series of events which results in activation of the entire complement system. The primary structure of C1q is unusual in that 40% of its amino acid sequence is composed of repeating X-Y-Gly triplets where X is often proline and Y often hydroxyproline or hydroxylysine, indicating a striking similarity to collagen^{1,2}. It has also been proposed that these collagen polypeptide-like stretches in C1q associate in triple helical fashion³. Because of the similarities between these portions of C1q and collagen the present investigation was undertaken to determine whether C1q is synthesised by collagen-producing cells, namely fibroblasts.

Three fibroblast lines were used: human lung fibroblasts (from 13-week embryos) obtained from Gibco-Biocult (Paisley, Scotland); human skin fibroblasts (from 14-16-week embryos) and rat skin fibroblasts (from 16-18-day embryos) cultured in this laboratory. All cell lines were grown in air on glass slides in Petri dishes, or in 50-cm² glass culture flasks in HEPES buffered minimum essential medium containing 10% foetal calf serum, 2 mM glutamine, 0.25 mM ascorbate, 0.025 μ M ferric nitrate and 50 units of penicillin and streptomycin ml⁻¹. All lines formed collagen fibres (demonstrated by reticulin staining) and contained prolyl hydroxylase. The cells used in all experiments had been subcultured four to six times.

C1q was localised in all three cell lines growing on glass slides, by an immunoperoxidase procedure⁴ using a monospecific rabbit antiserum to human C1q (Hoechst Hounslow, England) and to rat C1q. As shown in Fig. 1, fibroblasts bind antibody to C1q avidly. This reaction (Fig. 1) is specific

Fig. 1 Localisation of C1q in cultured human fibroblasts. The cells were treated with monospecific antibody to human C1q and goat anti-rabbit IgG peroxidase conjugate by an immunoperoxidase procedure⁵.



and indicates that C1q was present in the fibroblast cytoplasm of all cell lines as shown by the following control experiments. Fibroblasts overlaid by normal rabbit serum, or antiserum to C1q absorbed with pure C1q (ref. 5) or with fresh human or rat serum before processing through the immunohistochemical procedure failed to stain any of the fibroblast lines (Fig. 2). Antisera to C1q absorbed with heat-denatured normal serum (which renders C1q incapable of reacting with its antibody) did not abolish the reaction seen in Fig. 1. To exclude the possibility that antiserum to C1q cross reacts with collagen two further absorption experiments were done: previous absorption of antiserum to human C1q with neutral salt-soluble collagen failed to alter the reaction seen in Fig. 1; likewise antiserum to human C1q did not react in agar diffusion plates with a mixture of Type I and Type III procollagen purified from human embryonic skin⁶. On this evidence, therefore, it seems unlikely that anti-C1q was reacting with intracellular collagen antigen.

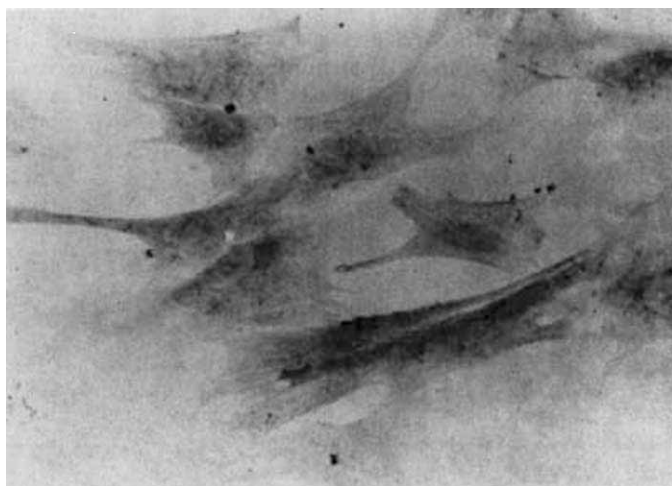


Fig. 2 Cells treated as in Fig. 1 except that normal rabbit serum was used instead of the antibody. No staining can be seen except that due to osmium tetroxide.

To determine whether C1q was also secreted by these cells, human fibroblasts from embryonic skin were grown to mid log phase under the same conditions in 50-cm² flasks. Each culture was then washed with 0.15 M NaCl (2 $\times$ 5 ml) and incubated in 10 ml of serum-free minimum essential medium (supplemented as above) containing 2.4 μ Ci ml⁻¹ of 3,4-³H-proline (specific activity 20 mCi mmol⁻¹) for 60 min at 37 °C. The cell layers were then washed with 7 ml of 0.15 M NaCl and the medium and NaCl wash pooled and frozen at -20 °C until used. The cells were collected in 0.15 M NaCl, resuspended to 10⁷ cells ml⁻¹ in 0.1 M phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.01 M

Table 1 Immunoprecipitation of C1q from human fibroblast cultures and medium

Experiment	Aliquot (ml)	C1q precipitated per 10 ⁷ cells* (c.p.m.)
Cell layer	0.15	1,407
Medium	2.5	4,225
	5.0	3,600

Human fibroblasts were cultured, pulsed with ³H-proline, collected, sonicated and centrifuged as described in the text. Labelled C1q was precipitated from 0.15 ml aliquots of the cell supernatants by the addition of 0.1 ml of fresh human plasma (as carrier C1q) and rabbit antiserum to C1q. Labelled C1q was precipitated from aliquots of the medium in a similar way (see text).

*The number of counts in the control tubes (with normal rabbit serum) was 20% and 5% of that found in those containing anti-C1q for the cell layer and medium respectively. All results are the average of duplicate estimations.

EDTA, sonicated and centrifuged at 100,000g for 90 min. Fresh normal human serum (as carrier C1q) was added to the supernatant and to medium dialysed exhaustively against phosphate-buffered saline containing 0.01 M EDTA. Rabbit anti-human C1q was then added in threefold excess to the cell supernatants and medium, incubated at 37 °C for 30 min and overnight at 4 °C. In control tubes normal rabbit serum was added in the same proportion as the rabbit antiserum to C1q. The immunoprecipitates were collected, washed, dissolved in 1 N NaOH, and the radioactivity in each precipitate counted in Bray's solution. As shown in Table 1, C1q is present not only in the cells themselves but is also rapidly secreted by them such that in a 1-h pulse period approximately three times more labelled C1q is present in the medium than in the cell layer. It should be noted that the total amount of protein present in the immune precipitate was constant. For example, in the quantitation of C1q in medium (Table 1) the total amount of protein in each precipitate was 0.39 mg and 0.33 mg using 2.5 and 5 ml of medium respectively. This, therefore, excludes the possibility that the radioactivity which coprecipitated with carrier C1q was due to nonspecific precipitation or absorption of tritium to the precipitate itself. These data also argue against the possibility that the reaction product seen in immunohistochemical experiments (Fig. 1) was due to absorption of preformed C1q from the serum containing medium by fibroblasts.

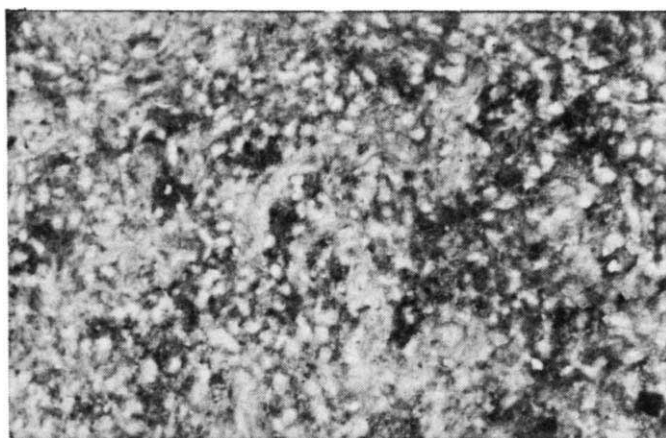


Fig. 3 C1q localisation in the cells of a silica granuloma. Granulomas were produced on the backs of adult albino rats by the subcutaneous injection of 1.0% suspension of Dornetrop quartz in saline. The granuloma illustrated was excised 12 d after its production; frozen sections were cut and C1q localised by the immunoperoxidase procedure⁵. There is a positive reaction around nuclei (clear holes) in many of the cells in the granulation tissue. It is possible that some of the reaction product is also extracellular, but it is difficult to resolve this point with certainty in this type of preparation.

In an attempt to demonstrate the origin of C1q *in vivo* various rat organs (including foetal and neonatal organs) were examined by the immunoperoxidase procedure as described above. This screen was entirely negative. C1q production, however, could be demonstrated *in vivo* in fibroblasts surrounding a silica granuloma which had been produced in the subcutaneous tissue of adult Wistar rats as shown in Fig. 3.

These data indicate that fibroblasts *in vitro* synthesise and secrete C1q. It seems likely that C1q is also produced by fibroblasts *in vivo* at least in pathological conditions where fibroblasts are functioning at a much higher rate than normal, namely in a silica granuloma. This evidence does not exclude C1q production by other cell types *in vivo* or *in vitro*⁷. It is noteworthy, however, that freshly isolated human blood lymphocytes, the only cells we have found

which do not contain prolyl hydroxylase *in vitro* (and presumably do not synthesise collagen) do not contain C1q when examined by the immunoperoxidase procedure.

These results also suggest that primary fibroblast cultures may serve as a useful cell source for studies of C1q biosynthesis.

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Detection of α -foetoprotein in mouse liver differentiated hepatocytes before their progression through S phase

α -FOETOPROTEIN (AFP) is a serum protein present in large amounts during embryonic life and preserved in only nanogram quantities in adult mammals¹. Increased levels of AFP are seen in adults in association with two forms of tumours—teratocarcinomas and hepatocarcinomas—and also during liver regeneration². In early development AFP is synthesised in the yolk sac and in the liver^{2,3} where it has been demonstrated by immunofluorescence in the majority of the hepatocytes⁴. Detailed aspects of AFP synthesis by hepatocytes, however, remain to be established. It has been suggested that it is synthesised by a definite population of hepatocytes⁵, by one of the stages in their differentiation^{6,7}, or by liver cell precursors⁸. Sufficient data to decide between these alternatives are not presently available. We describe here the immunohistochemical localisation of AFP in regenerating mouse livers, in which DNA-synthesising cells have been identified by thymidine incorporation. AFP was found in a minority of adult differentiated hepatocytes, many of which had not progressed through S phase. Thus DNA synthesis is not a prerequisite for the induction of AFP synthesis in the adult liver.

Liver regeneration was induced by CCl₄ inhalation⁹ in SWR or SWR/S mice (20–25 g). Serum AFP levels, as measured by immunodiffusion, increased significantly on day 2, reached a maximum on days 3–4 and had returned to basal levels by days 7–10. The centrolobular necrosis caused by CCl₄ resulted in practically all the remaining viable hepatocytes passing through one round of DNA synthesis, and a small fraction undergoing a second round. The most intensive DNA synthesis was found between 36 and 60 h and the duration of S phase in individual hepatocytes was 6.9 h (ref. 10). These values were similar to those obtained in liver regenerating after partial hepatectomy, $S=7.0 \pm 1.2$ h (ref. 11).

Mice with regenerating livers after exposure to CCl₄ were

Table 1 AFP in hepatocytes with and without thymidine incorporation

Mouse	1	2	3	4	5
% Labelled nuclei in total hepatocyte population	67 (^3H 55 + ^{14}C 12)	54 (^3H 42 + ^{14}C 12)	0.6 (^3H 0.3 + ^{14}C 0.3)	82	38
% Labelled nuclei in AFP-positive hepatocytes	78 (^3H 69 + ^{14}C 9)	34 (^3H 22 + ^{14}C 12)	4 (^3H 2.3 + ^{14}C 1.7)	82	64
Number of nuclei of AFP-positive hepatocytes examined	486	185	175	335	257

Mice exposed to CCl_4 (ref. 11) were injected intraperitoneally with radioactive thymidine in 0.1 ml saline as follows. Mouse 1: $1 \mu\text{Ci g}^{-1}$ methyl- ^3H -thymidine ($19.8 \text{ Ci mmol}^{-1}$, Isotope, USSR) every 6 h, with $1 \mu\text{Ci g}^{-1}$ methyl- ^{14}C -thymidine (39 mCi mmol^{-1} , Isotope, USSR) 1 h before being killed. Mice 2 and 3: $1 \mu\text{Ci g}^{-1}$ methyl- ^3H -thymidine ($20.5 \text{ Ci mmol}^{-1}$, Isotope, USSR) every 3–4 h, with $0.8 \mu\text{Ci g}^{-1}$ methyl- ^{14}C -thymidine (54 mCi mmol^{-1} , Prague) 1 h before being killed. Mice 4 and 5: $1 \mu\text{Ci g}^{-1}$ methyl- ^3H -thymidine ($20.5 \text{ Ci mmol}^{-1}$, Isotope, USSR) every 3–4 h, the last injection being given 1 h before being killed. Mouse 1 was killed 48 h after exposure and mice 2–5 after 42 h. Livers were fixed in cold ethanol-acetic acid¹². AFP was localised with indirect immunofluorescence on $3\text{-}\mu\text{m}$ liver sections, using monospecific antibodies to murine AFP isolated from rabbit anti-murine AFP antiserum. Staining could be completely blocked by the addition of an equivalent amount of purified murine AFP (ref. 12). AFP-positive hepatocytes were photographed, after which the sections were washed, dehydrated, dried and covered with photosensitive emulsion (N ii Chim Photo, Moscow, USSR). After exposure for 1 month at 4°C the autoradiographs were developed and the sections stained with haematoxylin-eosin. The percentage of nuclei labelled with ^3H or ^{14}C , distinguishable by grain density, were determined after counting 2,000 hepatocyte nuclei. Using the immunofluorescent photographs all AFP-positive cells were identified on corresponding autographs and nuclei with ^3H or ^{14}C label counted.

repeatedly injected with methyl- ^3H -thymidine and killed by decapitation on day 2, 1 h after the last injection of radioisotope. In some mice the final injection was of methyl- ^{14}C -thymidine, as described in Table 1. The livers were fixed immediately in cold ethanol-acetic acid and embedded in paraffin¹². AFP was localised in liver sections by indirect immunofluorescence, and the nuclei of the cells in which DNA synthesis had occurred were subsequently identified by autoradiography. Damaged cells which could accumulate serum proteins nonspecifically because of increased membrane permeability were detected by the immunohistochemical demonstration of the serum marker protein, IgG. Consecutive sections were used so that individual cells could be stained for both AFP and IgG (ref. 12).

Using indirect immunofluorescence we failed to find any AFP-containing structures in the liver sections of the control

normal adult mice. For the first time several AFP-positive hepatocytes were found in the liver section 24 h after the CCl_4 poisoning. During day 2 their amount and intensity of fluorescence increased rapidly, reached a peak on day 3 followed by a sharp decline on the day 4. On day 2, when the experiment was terminated, AFP was present in not more than 3–5% of the hepatocytes, in both mono- and binucleate cells. Most AFP-containing cells were found in the region adjacent to the centrolobular necrosis (Fig. 1), although in some mice they were present in the periportal area. Intense IgG fluorescence was observed in the necrotic centrolobular cells, demonstrating their uptake of serum proteins, but was absent in the great majority of AFP-positive cells. It does not therefore seem that the latter have stained because of nonspecific uptake of AFP due to elevated serum levels. In the following analysis of thymidine

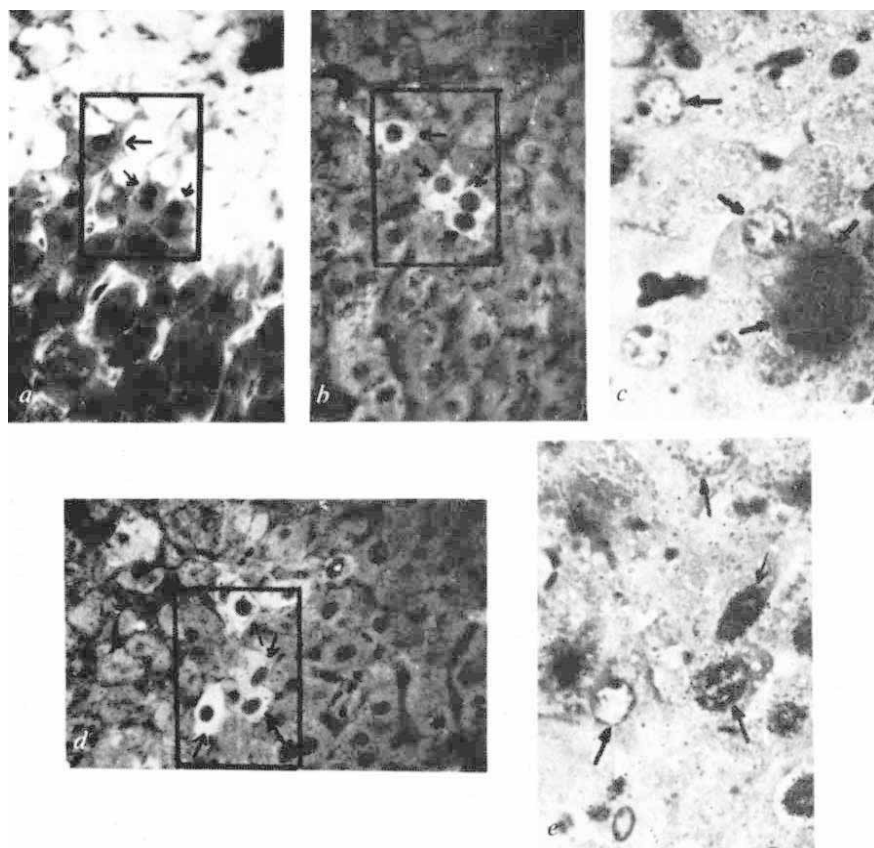


Fig. 1 AFP in the regenerating murine liver (mouse 1). Paraffin sections of liver fixed with ethanol-acetic acid and treated with rabbit anti-murine IgG serum (*a*) or purified rabbit antibodies anti-murine AFP (*b* and *d*) and FITC-conjugated donkey anti-murine IgG serum (Gamaleya Institute, Moscow, USSR). *c* and *e*, Autoradiographs of the areas ringed in *b* and *d*. Arrows show the same hepatocytes on the immunofluorescence photographs and on the autoradiographs. ^{14}C -label is easily distinguished from ^3H -label. *b* and *c*, Two nuclei of the AFP-positive hepatocytes are in S phase according to the intensive ^{14}C -label; two nuclei have no label. *d* and *e*, Two nuclei of the AFP-positive hepatocytes without label; two nuclei have incorporated ^3H -label and have progressed through S phase or all mitotic cycle. Magnification: *a*, *b* and *d* $\times 105$; *c* and *e* $\times 225$.

incorporation only IgG-negative, AFP-positive cells have been scored.

The percentage of labelled nuclei identified by autoradiography in the regenerating livers showed marked individual variation (Table 1). In all livers examined, however, numerous AFP-positive cells were seen without labelled nuclei (Fig. 1), as well as cells labelled in S phase with ^{14}C -thymidine 1 h before killing and cells labelled with ^3H -thymidine which had already progressed through S phase. The unlabelled cells comprised between 18 and 96% of the total of AFP-positive hepatocytes (Table 1).

The mice were injected with radioactive thymidine at intervals of 6 h (mouse 1) or 3–4 h (mice 2–5), that is less than the mean duration of S phase in these hepatocytes. As the thymidine was present in the circulation for at least 1 h after each injection, it is therefore unlikely that any DNA-synthesising cells could have escaped incorporation. Evidently AFP is localised in cells that have not yet commenced DNA synthesis during regeneration.

The time of appearance of the small number of AFP-positive hepatocytes, their quantity and intensity of fluorescence, all correlated directly with the changes in serum AFP measured. These observations, together with the failure of most AFP-positive cells to show nonspecific uptake of serum proteins, suggests that AFP is synthesised in these cells. If this is so, then the results show that AFP synthesis may be induced in the regenerating mouse liver in adult differentiated hepatocytes before the beginning of DNA synthesis. A similar suggestion has recently been made by Watanabe *et al.*¹³, who have reported that serum AFP levels increase before the onset of proliferation in livers of rats treated with ethionine. In this context, it is worth noting that in an unrelated system (cultured erythroleukaemic cells), globin synthesis can be induced by butyric acid in the absence of cell division¹⁴.

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Interaction of lighting and other environmental variables on activity of hypothalamo–hypophyseal–gonadal system

SINCE the pioneer study of Rowan¹, environmental lighting has been considered a predominant regulator of breeding activity in a variety of birds. A long daily photoperiod increases the levels of stored and circulating gonadotropins, and accelerates gonadal growth in Japanese quail^{2,3}, white-crowned sparrow⁴, American tree sparrow⁵, domestic pigeon⁶ and mallard⁷. Other environmental variables such as temperature, rainfall and courtship are also known to influence breeding⁸. The nature of the endocrine response to temperature and rainfall is unknown, but the effect of male courtship on female endocrine systems has been studied extensively in ring doves (*Streptopelia risoria*). It increases the plasma levels of oestrogen⁹, luteinising hormone¹⁰ (LH) and progesterone¹¹, which in turn, induce sequential behavioural changes in the female that culminate in egg laying^{12,13}. Using ring doves, I have investigated how environmental light and courtship interact to elicit the species-specific endocrine response. Since there is no significant change in testis size or weight during various stages of the breeding cycle of doves, this is not a suitable criterion for assessing the activity of the male endocrine state. Instead I used compensatory testicular hypertrophy, which is a response to environmental lighting in many species of birds, and investigated whether long daily photoperiod was required for this phenomenon in male ring doves, and whether courtship had any effect on this response.

Table 1 Description of experimental groups

Group	No. of males	Courtship condition	Daily light-dark (LD) cycle (h)
1	10	Isolation*	14:10
2	10	Isolation	8:16
3	10	Brief courtship†	14:10
4	10	Brief courtship	8:16
5	10	Breeding‡	14:10

* Males were housed individually in isolation rooms where they could hear, but not see, other birds.

† Males were introduced twice to a stimulus female in breeding cages that contained nest bowl and nest materials. Each exposure lasted 20 min. Sessions were spaced 1 week apart.

‡ Males were in a breeding cage that contained a stimulus female, nest bowl and nest materials throughout the 4-week experimental period.

Sixty reproductively experienced male ring doves from the indoor colony of the Institute of Animal Behavior, Rutgers University, were used. The colony is maintained under LD 14:10 controlled photoperiod and $21 \pm 1^\circ\text{C}$ temperature. Fifty of the 60 birds were anaesthetised intramuscularly with equithesium (0.22 ml per 100 g), and the (larger) right testis of each was removed and weighed. The birds were then randomly assigned to one of five groups (Table 1), characterised in terms of daily photoperiod (LD 14:10 or LD 8:16) and the condition of courtship (isolation or breeding cage which contained a stimulus female). The remaining 10 doves were similarly anaesthetised, and both left and right testes removed and weighed. The stimulus females used for courtship condition were normal doves which showed sexual crouch, wing-flip and nest-coo¹⁴.

Four weeks after dextral castration, all 50 males were laparotomised. The left testis (LT) of each was removed, and its weight expressed as a percentage of that of the right testis (RT). Since the LT weight of intact male doves kept under LD 14:10 was, on average, 80% that of the RT (Table 2), I considered $\text{LT} \geq 80\%$ of RT as evidence for compensatory

Table 2 Compensatory hypertrophy of left testis after removal of right testis in the ring dove (*Streptopelia risoria*)

Group	Daily photostimulation/ courtship condition	No. of birds	Mean $\pm$ s.d. body weight (gm)	Mean $\pm$ s.d. right testis weight (mg)	Mean $\pm$ s.d. left testis weight (mg)	Left testis $\times 100$		Compensatory hypertrophy
						Right testis Mean	Range	
1	14 h L	10	160 $\pm$ 5	5,776 $\pm$ 11	5,737 $\pm$ 15	100†	85–114	Yes
2	Isolation	10	161 $\pm$ 8	6,278 $\pm$ 9	4,026 $\pm$ 11	60*	30–74	No
3	8 h L	10	147 $\pm$ 8	5,741 $\pm$ 8	5,766 $\pm$ 20	101†	96–109	Yes
4	Brief courtship	10	156 $\pm$ 7	5,580 $\pm$ 14	6,723 $\pm$ 26	112†	94–130	Yes
5	8 h L	10	159 $\pm$ 6	5,780 $\pm$ 9	5,323 $\pm$ 32	96	83–120	Yes
C	Brief courtship	10	158 $\pm$ 6	5,661 $\pm$ 12	4,632 $\pm$ 17	80	75–86	
	14 h L							
	Breeding							
	Isolation control							

Analysis of variance for the randomised groups: $F = 3.52$, $\alpha = 0.05$.

* t test significant ($P < 0.01$) only between group 2 and every other group.

† t test significant ($P < 0.01$) in comparison with group C.

‡ Measurements were taken 4 weeks after dextral castration.

hypertrophy. The results are summarised in Table 2. (1) Compensatory testicular hypertrophy occurred in birds under LD 14:10 (groups 1, 3 and 5), but not in birds kept isolated and under LD 8:16 (group 2); (2) LT weights of birds in group 2 averaged only 60% that of RT, indicating that the LT had regressed rather than augmented. (An histological study showed an increase in size of seminiferous tubules, Leydig cells and sperm count in the augmented testis.)

These results, together with the finding that LD 8:16 caused testicular regression in ring doves (unpublished) and the fact that other species of birds show a decline in plasma LH level^{3–5}, demonstrate that long daily photoperiod is an important regulating factor for the feedback function of the hypothalamo-hypophyseal-gonadal system. Short exposure to courtship (two pairing sessions with stimulus females for 20 min each and 1 week apart) however, resulted in good testicular hypertrophy in male doves kept under LD 8:16 (group 4). The stimulatory effect of courtship on male doves does not seem to increase with increased duration of exposure to stimulus females. There was no apparent difference in LT weight among birds kept under LD 14:10, regardless of whether the birds were isolated or exposed to stimulus females (groups 1, 3 and 5). Thus, it seems that the combined effects of courtship and lighting on the hypothalamo-hypophyseal-gonadal system are guided by an adaptive change of endocrine state for the species, rather than simply a mathematical sum of respective environmental effects.

Two brief exposures to a stimulus female (20 min each and 1 week apart, group 4) affected males in a manner comparable with that of 6 h additional daily photoperiod for 4 weeks (group 1); this makes the stimulatory effect of female courtship on compensatory testicular hypertrophy even more significant. This is equally true of the effect of male courtship on female systems.

Under long photoperiod (LD 14:10), follicles of female ring doves took 20 weeks to develop from 2–3 mm to 8–9 mm in diameter, and they then became atresic. In contrast, when paired with male doves in breeding cages, female ring doves of similar ovarian stage (follicle size 2–3 mm) took only 7–11 d to develop large follicles (14–15 mm) which subsequently ovulated and oviposited (unpublished).

In male and female Japanese quail, although long photoperiod increases gonadotropin-releasing factor in the posterior basal hypothalamus area^{3,15}, there is no direct evidence to suggest a similar effect of courtship stimulation on releasing factor by the hypothalamus. In my study, compensatory hypertrophy in birds kept under LD 8:16 and exposed to stimulus females twice (group 4), strongly suggests that courtship stimulates either: gonadotropin synthesis, gonadotropin release, or the sensitivity of the pituitary to gonadotropin-releasing factors. I favour the second possibility in view of the following findings in the

Japanese quail: (1) Long daily photostimulation results in gonadal development³ and hypothalamic gonadotropin-releasing activity¹⁵; and (2) a change of daily photoperiod from LD 14:10 to LD 8:16 results in a lower level of plasma LH and testicular regression, but a higher level of pituitary LH; these events occurred within 6–16 d following the onset of short photoperiod³. Similarly, in male golden hamsters, exposure to non-stimulatory photoperiod does not alter the increased pituitary LH and follicle stimulating hormone (FSH) content after castration, whereas the serum levels of LH and FSH decline substantially¹⁶.

On the basis of these findings, it seems that environmental photoperiod affects the synthesis as well as release of gonadotropins; the releasing mechanism being more responsive to an immediate photoperiodic change than the synthesis, whereas synthesis (and/or store) is regulated by a long term photoperiodic change and endogenous feedback system. The failure of the hypertrophy response in birds kept in short light regimes (group 2) is thus a result of the cessation of gonadotropin release in response to the short photoperiod. Gonadotropin release can, however, be induced by other environmental stimulation (such as a female stimulus, warm temperature, rainfall and so on), which results in the hypertrophy seen in group 4 birds kept in short light regimes. The release is possible since synthesis (and/or store) of gonadotropin is not affected by immediate photoperiodic change.

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A discontinuous relationship between the acetylcholine-activated channel conductance and temperature

SINCE the introduction of acetylcholine (ACh) 'noise' analysis^{1,2}, it has been shown that several manipulations affect ACh-receptor molecular events³⁻⁵. The ACh receptor is an integral membrane protein and is therefore intimately associated with surrounding membrane lipids. We have begun experiments using embryonic chick muscle in culture to determine whether surrounding lipids affect the function of the ACh receptor. As a first step, we have analysed ACh current noise to determine the temperature dependence of the mean single channel conductance ($\bar{g}_o$) and open time ($\bar{\tau}_o$).

At frog endplates $\bar{\tau}_o$ is gradually prolonged as the temperature (T) is lowered but $\bar{g}_o$ remains essentially unchanged. We observed a similar relation between $\bar{\tau}_o$ and T in cultured chick muscle, but in contrast to results obtained in amphibia, $\bar{g}_o$ was markedly decreased at low temperatures. Moreover, there was a marked discontinuity in the relation between T and $\bar{g}_o$. A clear 'transition' temperature was observed at $\sim 20^\circ\text{C}$ which suggests that $\bar{g}_o$ may depend on the 'fluidity' of the receptor microenvironment.

ACh-induced transmembrane currents were recorded in voltage clamped large spherical muscle cells (Fig. 1). Such spheres or 'myoballs' are more suitable than elongated cylindrical muscle fibres for voltage clamp analysis. The interior of each myoball is isopotential and the membrane potential can be held constant, even in the face of large ACh responses (up to 200 nA). Myoballs are 'normal' in the sense that they are extremely sensitive to ACh and they contract.

Control of membrane potential was achieved with two intracellular electrodes—one for measuring the membrane potential and the other for supplying the feedback current required to hold the membrane potential constant. The microelectrodes had a resistance of 3–10 M Ω when filled with 3 M KCl. A microelectrode filled with ACh was located 50–100 μm away from the cell and ACh was ejected by prolonged, positive pulses. Temperature was controlled by perfusing ethanol ($\pm 60^\circ\text{C}$ to -30°C) between the glass bottom of the recording chamber and the objective of the inverted microscope. In most

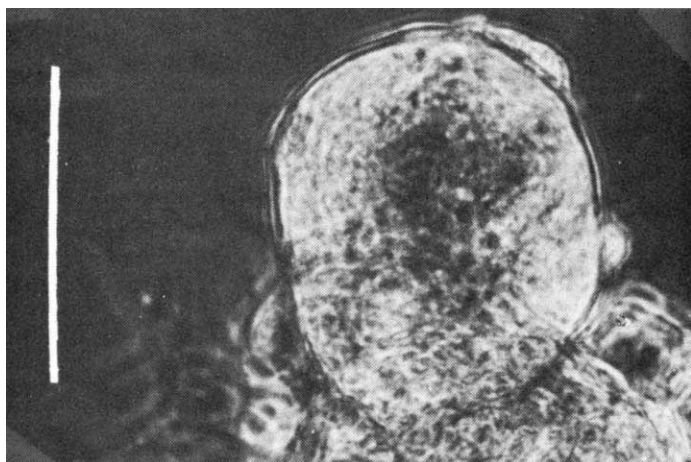


Fig. 1 A large chick myoball. Spheres were obtained by adding mononucleated muscle precursor cells dissociated from 11-d pectoral muscles to Petri dishes coated with Sylgard (Dow Corning). The cells did not attach to this surface, and over the next 2–3 d muscle precursor cells fused with one another in suspension to form multinucleated 'myoballs', 50–120 μm in diameter. For electrophysiological experiments, myoballs were allowed to settle on collagen-coated glass coverslips which were then placed in a chamber on an inverted phase contrast microscope. Smooth surfaced myoballs could be easily distinguished from aggregates of fibroblasts which had a cobblestone-like appearance. Bar = 100 μm .

experiments, the membrane potential was clamped at -80 mV . Feedback currents were stored on magnetic tape (frequency response 0–1,250 Hz).

A typical ACh response is shown in Fig. 2a. The horizontal bar indicates the duration of the ACh pulse. The lower trace is a low gain d.c. recording of the membrane ionic current and the upper trace is a parallel, high gain filtered (bandpass 1–200 Hz) recording which shows the fluctuations or noise of the ACh-induced current. In this example, the maximum inward current produced by ACh was 100 nA.

ACh responses were analysed with a PDP/8e computer. The single-channel conductance, $\bar{g}_o$, was estimated from the slope

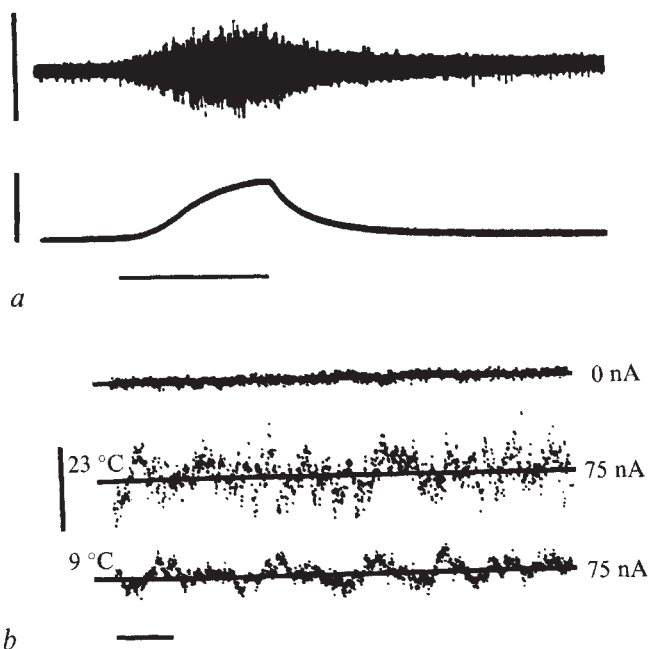


Fig. 2 ACh-induced membrane currents in voltage clamped myoballs. *a*, A 15-s long pulse of ACh (horizontal bar) results in a large increase in mean membrane current (lower trace: a low-gain d.c. recording) and an increase in current fluctuations (upper trace: a parallel high gain filtered channel). The membrane potential was clamped to -95 mV . The slow rise of the response reflects the time required for ACh to diffuse from the ACh pipette located 50 μm away. Vertical bars: 3 nA upper trace, 100 nA lower trace. *b*, Digitalised, 2-s long, samples of ACh current fluctuations (noise). The upper trace shows control fluctuations in the absence of ACh. The steady holding current is defined as zero. ACh responses obtained at 23 and 9°C are shown in the middle and lower traces respectively. The mean ACh current was 75 nA at both temperatures. Note that at 9°C , high frequency components are less prominent and that the amplitude of the noise is decreased. Vertical bar, 2 nA; horizontal bar = 250 ms.

of the linear relation between the variance of current fluctuations and the mean membrane current. The mean channel open time, $\bar{\tau}_o$, was estimated from the spectral density function, $S(f)$; $\bar{\tau}_o = 1/2\pi f_c$ where f_c is that frequency at which $S(f)$ is reduced by half^{1,2,6}. At 37°C , when the membrane potential was set between -70 and -95 mV , $\bar{g}_o$ ranged between 25 and 45 pmho, and $\bar{\tau}_o$ between 2.5 and 4.0 ms.

Figure 2b shows digitalised records of control or background noise and ACh-induced noise at two temperatures (sampling rate: 500 s^{-1}). As reported in previous studies in amphibia, lower frequency components predominate at the lower temperature. In this example, $\bar{\tau}_o$ increased from 7.2 ms at 23°C to 16.1 ms at 9°C . In these chick cells, $\bar{\tau}_o$ increased continuously as T was lowered from 37 to 7°C . In addition, Fig. 2 shows that the amplitude of the current fluctuations is markedly decreased at 9°C , even though the mean ACh-induced current is the same at the two temperatures. Estimates of $\bar{g}_o$ in this cell decreased

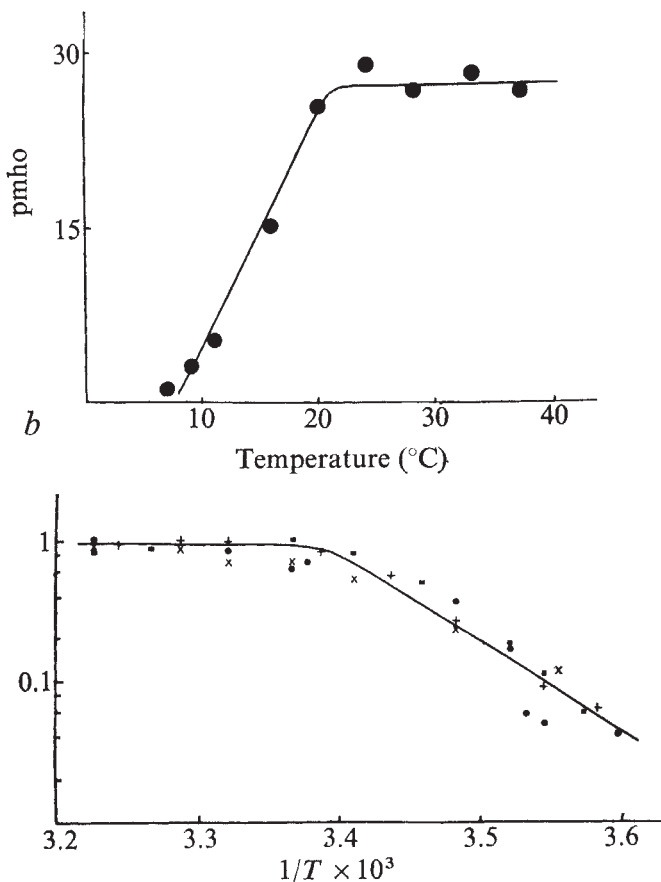


Fig. 3 Temperature dependence of single channel conductance $\bar{g}_o$ in chick myoballs. *a*, Nine ACh responses elicited in the same cell at different temperatures between 7 and 37 °C are shown. The relationship between $\bar{g}_o$ and temperature is discontinuous; a sharp transition is observed at ~ 20 °C. *b*, Arrhenius plot of normalised data obtained in four different myoballs. The activation energy (E_a), calculated from the equation $\log \bar{g}_o = C - (E_a/2.3R)(1/T)$ (R = gas constant, T = absolute temperature, and C = a constant) increases from virtually 0 above 20 °C to 30 kcalorie mol^{-1} below this critical temperature.

from 31 pmho at 23 °C to 7.6 pmho at 9 °C. A comparable or larger decrease in $\bar{g}_o$ was obtained whenever the temperature was lowered to 10 °C, and in each case the change in $\bar{g}_o$ was completely reversed on rewarming the preparation. This dramatic effect cannot be explained by a negative shift in the ACh reversal potential (measured by ACh application at membrane potentials between -80 and $+30$ mV) which remained fixed at ~ -5 mV between 37 and 5 °C.

In contrast to the continuous relationship between $\bar{t}_o$ and T , there was a clear discontinuity in the relation between $\bar{g}_o$ and T in every case examined (Fig. 3). There was no significant decrease in $\bar{g}_o$ as T was lowered from 37 to 20 °C. As T was lowered further to 5 °C, however, there was a nearly tenfold decrease in $\bar{g}_o$. An Arrhenius plot including points from four myoballs is shown in Fig. 3*b*. From the slope it can be determined that the activation energy for those factors or events which determine the magnitude to which an ACh channel can open is increased by 30 kcalorie mol^{-1} at ~ 20 °C.

A transition temperature has been observed in studies of other membrane proteins where enzyme activity, or transport or mobility within the lipid bilayer were assayed^{7,8}. It is thought that a change in membrane fluidity occurs at the critical temperature. That is, above the critical temperature, the phospholipid fatty acid side chains are flexible and the interior of the membrane is fluid; below the critical temperature, movements of fatty acid side chains are restricted and the membrane core is more solid. It is plausible that the same mechanism explains the discontinuous relation between $\bar{g}_o$ and T described

in this report. The degree to which an ACh-activated channel opens may depend on the flexibility of fatty acids in its micro-environment. It may be significant that the membranes of poikilothermic animals (in which $\bar{g}_o$ remains nearly constant between 10 and 23 °C) contain relatively more unsaturated fatty acids which 'melt' at a lower temperature⁹. The fact that no clear break was observed in the relation between $\bar{t}_o$ and T may simply mean that different molecular processes regulate $\bar{t}_o$ and $\bar{g}_o$.

It should be possible systematically to modify the membrane lipids of rapidly growing muscle cells by adding specific fatty acids to a culture medium made up with delipidated serum¹⁰. The relationship between $\bar{g}_o$ and membrane fluidity might then be defined in more detail. In addition, changes in regional and/or overall membrane fluidity may alter the degradation of ACh receptors or their insertion into, or distribution within, the membrane.

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Long term potentiation is accompanied by a reduction in dendritic responsiveness to glutamic acid

HIPPOCAMPAL synapses show an unusual degree of physiological plasticity. Relatively modest levels of repetitive stimulation cause considerable enhancement in subsequent responses to single pulse stimulation, and this potentiation lasts for hours and even days¹⁻⁶. These findings, which have now been replicated in several laboratories, have aroused much interest, first because they seem to represent an excellent starting point for the analysis of synaptic plasticity in mammalian brain, and second, because their rapid development and persistence suggest that they may be related to processes involved in behavioural plasticity. The studies reported here represent an attempt to analyse the mechanisms underlying physiological plasticity in the hippocampus, in particular the locus and anatomical specificity of the effect. The change in magnitude of response which occurs after repetitive stimulation could reflect either pre-synaptic or postsynaptic adjustment. If the latter were the case, the change might involve a particular dendritic region innervated by the stimulated input, or alteration in the status of the entire postsynaptic neurone. Seeking data relevant to these questions, we have measured the effects of glutamic acid applied iontophoretically to different levels

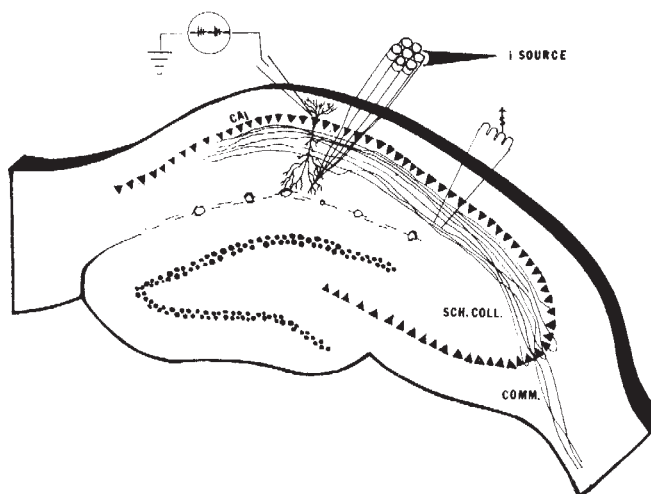


Fig. 1 Schematic representation of the transverse hippocampal slice and the experimental electrode arrangement. Extracellular unit activity and evoked potentials were recorded from the CA 1 pyramidal cell somal layer as indicated. The separate multi-barrelled iontophoresis electrode could be placed at any point in the apical and basal dendritic zones of a particular neurone for the purpose of glutamic acid ejections. Schaffer collateral-commissural stimulation was accomplished through a bipolar electrode placed along the tract at the CA 1-CA 2 border.

of the dendritic trees of the pyramidal cells before and after potentiation of the extracellular postsynaptic response.

Glutamate more or less universally excites neurones⁷⁻⁹ and it is generally thought that it does this by depolarising the cell through an action on sodium and possibly potassium channels⁹. We reasoned that if potentiation produced a lasting change in the membrane properties of the postsynaptic cell, this might cause changes in that cell's response to discrete applications of glutamic acid. Furthermore, if the postsynaptic effect were generalised, that is, not restricted to the dendritic area affected by the driven input, then an altered response would be found to glutamic acid applied anywhere in the dendritic field. To test these possibilities we have used *in vitro* transverse slices of rat hippocampus, prepared and maintained as described before¹⁰. The experimental arrangement is described in Fig. 1. Briefly, a bipolar stimulating electrode (62 μm wire) was placed on the Schaffer collaterals (the fibre connection consisting of collateral axons from the CA 3 field to the CA 1 field of pyramidal cells (Fig. 1)) and a recording micropipette was guided into the layer of pyramidal cell bodies, the proximal dendrites of which receive the Schaffer input. The recording electrode was advanced until it was located next to a spontaneously active cell. The electrode recorded both the spike activity of the "isolated" cell and the characteristic field potential elicited by stimulation of the Schaffer collaterals. This response is a simple monophasic positive wave when threshold stimulation (4-7 V) is used; at slightly higher voltages a sharp negative deflection is superimposed on the wave (Fig. 2). This negative wave has been shown by Andersen and associates to be an envelope of synchronously driven cell spikes, that is, in their words, a "population spike"¹¹. We used this response as our index of the magnitude of the postsynaptic response. A third multi-barrelled electrode was used for iontophoretic application of 0.2 M L-glutamate to various regions of the pyramidal cells' dendritic field. Only a single CA 1 dendritic locus was tested in each slice. When the recording and electrical stimulation electrodes were in position the iontophoresis electrode was lowered into the chosen region of the dendritic field while glutamate was ejected at 20-30 nA until the recorded cell was vigorously, though not maximally, driven. The iontophoresis current was then lowered until a short latency and stable response were obtained for a 10-s test period. When all three electrodes were in position the experiment was

begun (usually at about 2 h after preparation of the slices). A constant current source was programmed to pulse the glutamate on to the CA 1 dendrites for 10 s every 40 s for 3-5 min. During this time averages of 10 field potentials were collected in response to stimulation of the Schaffer collateral bundle. Stimulus pulses were applied once every 5 s at a voltage adequate to elicit a small (less than 1-mV) population spike. Then 15-Hz tetanic stimulation was delivered for 15 s, after which iontophoretic applications were conducted as before. Field potentials were again collected and averaged 1, 4, 7, 12, and 20 min after 15-Hz stimulation—these were elicited by the same voltage and frequency as used before the conditioning train.

Twenty-two slices were tested by this procedure. Four of these did not show potentiation of the "population spike"

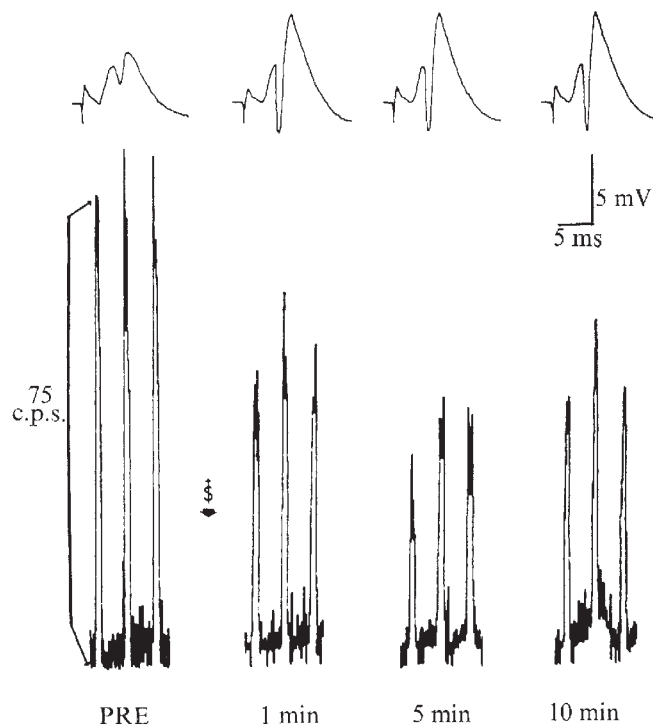


Fig. 2 The upper traces are computer averages of the field potential recorded by the pipette located in the cell body layer before (Pre) and 1, 5 and 10 min after a 15-Hz, 15-s train of stimulation pulses (denoted by S) to the Schaffer collateral system. The field potential and the population spike, the negative deflection riding on the positive wave, were greatly enhanced by the tetanising stimulation. The lower traces are records of the rate of spike discharges of a small population of pyramidal cells, recorded by the same microelectrode used to collect the field potentials, in response to a 10-s iontophoretic pulse (25 nA) of glutamic acid applied in apical dendritic field 200 μm away from the cell layer. Repetitive stimulation caused a decrease in the excitation produced by the amino acid.

after the conditioning pulses and in two others the degree of response facilitation was quite modest. In the rest of the slices, the "post-tetani" responses were at least twice the amplitude of the control responses. In a few slices from this last group the potentiation was transient and the response was clearly returning towards baseline by the end of the test runs.

The effects of the tetanising stimulation on the unitary response to glutamic acid were consistently the reverse of that displayed by the field potential. In addition it seemed that the nature and degree of the decrease in response to glutamic acid were related to the duration and magnitude of field potential potentiation. In slices which showed a potentiated response the excitation elicited by glutamic acid was reduced by at least 25%. The effect appeared within 30 s of the termination of the conditioning train and

generally persisted throughout testing. Furthermore, in those slices in which the potentiation was transient the reduction of the response to glutamic acid gradually dissipated (Fig. 3).

As mentioned, two slices showed very modest potentiation and in these cases the suppression of glutamic acid excitation was less than 25%. Finally, three of four slices which did not potentiate also did not exhibit any change in responsiveness to iontophoretically applied glutamic acid. The one exception demonstrated a very slight and transient reduction. Table 1 summarises these points. In 10 of the slices we used a second train of conditioning pulses and measured the effects of this on the response of the pyramidal cells to glutamic acid. The results of these tests were comparable with those obtained after the first tetanus: that is a strong potentiation ($N=6$) was accompanied by a reduction in responsiveness to glutamic acid, whereas absence of potentiation ($N=4$) was correlated with essentially no change in iontophoretically induced unitary excitation (Table 1, bottom).

Glutamate has been found (ref. 12 and unpublished results of V.K.G., H. J. Spencer, C. W. Cotman and G.S.L.) to excite pyramidal cells more or less independently of where it is applied to the dendritic tree, and our study confirmed this. More pertinent to our findings, depression of glutamic acid-induced excitation was found even when the amino acid was applied outside the region of Schaffer collateral innervation (to the basal dendrites), or to the apical dendritic tips in the zone of entorhinal innervation (Fig. 1).

Fig. 3 An example of post-tetanic potentiation which did not persist for more than 15 min after the 15-Hz, 15-s conditioning train (S). Note the reduction of glutamic acid effect while the field response was in a potentiated state, and the return of driving to preconditioning levels as the evoked potential decreased to baseline levels. All variables are the same as in Fig. 2.

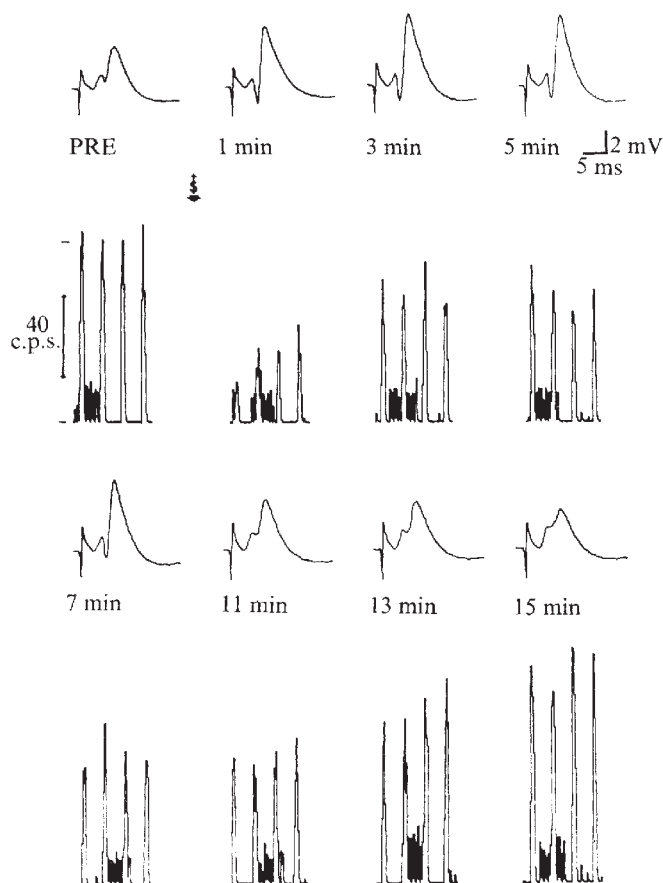


Table 1 Potentiation and response to glutamate

	Relative degree of potentiation			
	0	1	2	
Relative depression of responses to glutamate	0	3	—	First test
	1	1	2	
	2	—	—	
	3	—	—	
	0	2	—	Second test
	1	1	1	
	2	—	—	
	3	—	—	

The figures are the numbers of slices displaying the noted degree of potentiation (0, no change; 1, less than 100% increase in population spike within 1 min of the end of stimulation train; 2, greater than 100% increase in population spike in this time frame) and a particular degree of reduction in response to iontophoretic application of glutamic acid (0, no change; 1, 10–25% reduction of firing rate; 2, 25–40% reduction in firing rate; 3, greater than 40% reduction). First and second tests are explained in text.

These results indicate that tetanic stimulation produces a lasting change in responsivity of the postsynaptic membrane and that this change is generalised to the entire neurone. The similarity in degree and time course of the alteration in glutamic acid responsiveness and the size and duration of potentiation suggest that these two phenomena are related. Postsynaptic depression after tetani has been reported in the peripheral nervous system (for example ref. 13) but the rapid development and persistent nature of the effects reported here make it unlikely that comparable processes are involved. Speculation as to the physiological nature of the changes responsible for the altered glutamic acid response will be more profitable after completion of intracellular recording studies which are currently in progress.

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Inhibitory postsynaptic current in voltage-clamped crayfish muscle

THE current-voltage relationship provides important information about the mechanism of ionic permeation through membranes. In excitatory synapses the relationship between membrane potential and excitatory postsynaptic current (e.p.s.c.) has been extensively studied using the voltage-clamp method^{1–6}. In the frog endplate it has been shown that the e.p.s.c. was smaller than would be expected from a linear

relationship during hyperpolarisation of the membrane^{3,4}. On the other hand, after iontophoretic application of acetylcholine, the synaptic current increased more than expected at hyperpolarised membrane potentials⁷. In investigating the current-voltage relationship it is important to measure the current on both sides of the equilibrium potential. Inhibitory postsynaptic current (i.p.s.c.) is more suitable for this purpose, because the reversal potential is near the resting potential and so the current-voltage relationship on both sides of the reversal potential can be measured using relatively small potential changes. Little attention has been paid, however, to inhibitory postsynaptic membranes, possibly because the inhibitory synapses are distributed diffusely over the surface of the cell or situated on dendrites remote from the cell body where the electrical changes are measured. In these conditions it is difficult to measure accurately the current-voltage relationship. We have cannulated the opener muscle of the claw in the crayfish (*Cambarus clarkii*) using a stainless steel wire, the membrane potential being clamped at various levels as described previously⁶. Although the inhibitory synapses are distributed over the whole surface of the muscle fibre, the space clamp condition of the muscle fibre was maintained well using this technique. We have found that the relationship between membrane potential and i.p.s.c. was highly nonlinear on hyperpolarisation of the membrane.

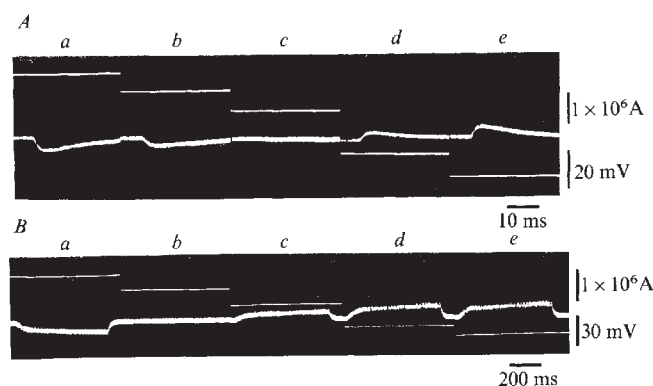


Fig. 1 Inhibitory postsynaptic current (i.p.s.c.) recorded at various membrane potentials. *A*, Superimposed i.p.s.c. at stimulation rate of 16 Hz: *a*, -37 mV; *b*, -46 mV; *c*, -57 mV; *d*, -79 mV; *e*, 90 mV. Upward deflection indicates the inside current. *B*, Train of i.p.s.c.s produced at 107 Hz: *a*, -47 mV; *b*, -60 mV; *c*, -76 mV; *d*, -99 mV; *e*, -109 mV.

The inhibitory axon was dissected at the melopodite and stimulated using a pair of silver electrodes. Inhibitory stimulation produced the inwardly directed i.p.s.c. at hyperpolarised membrane potentials (Fig. 1*A*, *d* and *e*). Although the time course of the i.p.s.c. was rather variable from fibre to fibre, the current reached its peak within ~4–5 ms and thereafter decayed almost exponentially with a time constant of 10–20 ms (total duration up to 50 ms). On depolarisation of the membrane, the amplitude of the i.p.s.c. decreased and the current reversed its sign at about -60 mV (Fig. 1*A*, *c*). The peak time and falling phase of i.p.s.c. at depolarised membrane potentials (downward deflections in Fig. 1*A*, *a* and *b*) were slightly longer than those at hyperpolarised membrane potentials. The time constant of the falling phase was 15.6 ± 5.1 ms at about -100 mV and 21.3 ± 6.0 ms at -40 mV (mean $\pm$ s.d., $n = 8$).

The time course of changes in i.p.s.c. was much slower than that of the e.p.s.c. recorded from the same muscle; the peak time of e.p.s.c. being about 2–3 ms and the time constant of the falling phase being 3.3 ± 1.1 ms at -100 mV. On repeated stimulation of the inhibitory axon, the falling phases of the i.p.s.c. summed up to a plateau (Fig. 1*B*). Such a summation was, however, not manifest in the e.p.s.c. (ref. 6).

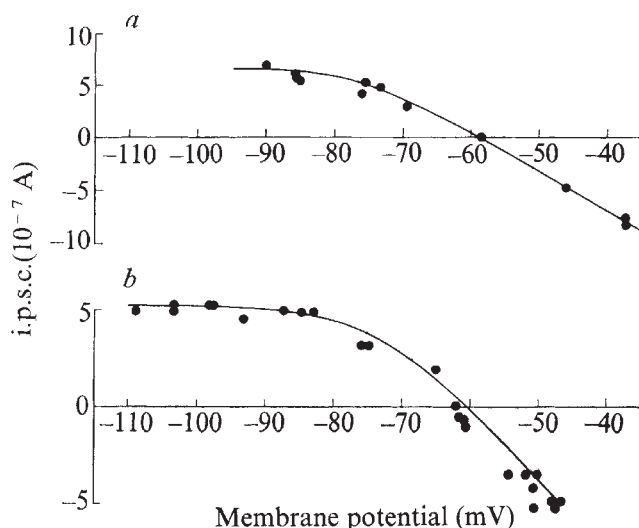


Fig. 2 Relationship between clamped membrane potential and amplitude of i.p.s.c. *a*, Peak amplitude of i.p.s.c. produced at 16 Hz; *b*, plateau level of i.p.s.c.s produced at 107 Hz. Inside current expressed as positive.

The peak amplitude of a single i.p.s.c. (Fig. 2*a*) and the plateau level of repeated i.p.s.c.s (Fig. 2*b*) were plotted against the clamped membrane potential. The synaptic current-membrane potential relationship was almost linear for the depolarised membrane up to -30 or -15 mV. When the membrane potential was hyperpolarised by about 20–30 mV from the reversal potential, however, the relationship deviated from linearity and the i.p.s.c. reached an approximately limiting value (Fig. 2*b*). This is different from observations of e.p.s.c., which increased almost linearly up to -120 to -150 mV (ref. 6). Since the i.p.s.c. is carried by Cl^- (ref. 8), the outwardly directed Cl^- flux seems to be limited at hyperpolarised membrane potentials. An essentially similar relationship was observed when GABA was added to the bath solution, and the increase in the membrane current at various membrane potentials measured using the voltage-clamp technique.

Since the time course of changes in i.p.s.c. became faster on hyperpolarisation of the membrane, nonlinearity may be explained by the voltage-dependent rate constants for the opening and closing of the conductance channels⁹. In many cases, however, the current-voltage relationship deviated more markedly from linearity than would be expected from the dependence of rate constants on voltage. Qualitatively similar nonlinearity—by the depolarisation of the membrane—has been observed at acetylcholine receptors in the frog endplate⁷ and electroplaque¹⁰; these could be accounted for using the above hypothesis.

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α -adrenergic receptors and pacemaker current in cardiac Purkinje fibres

IN recent years, membrane phenomena in cardiac fibres have been analysed with increasing accuracy, by the use of electronic feedback. Ionic transmembrane currents have now been measured and analysed for their rectifier properties, their time constants and their activation ranges. One of the current components analysed in much detail is the pacemaker current in cardiac Purkinje fibres. Evidence is now accumulating that this and other current systems are related to adrenergic receptors. Strong evidence has been put forward that this current is dependent on β -adrenergic receptors. In this paper we report results of tests carried out to investigate the involvement of α -adrenoceptors in controlling this process and to show that they are not directly involved.

The pacemaker potential in cardiac Purkinje fibres is generated by a time-dependent deactivation of a slow outward current, $i_{K2}^{1,2}$, which allows the time-independent steady-state inward current to depolarise the membrane. Using the formalism of Hodgkin and Huxley³ this current component is described in the following way

$$i_{K2} = \bar{i}_{K2} \times s \quad (1)$$

where $\bar{i}_{K2}$ is the instantaneous or fully activated current-voltage relationship and s is a dimensionless variable which controls the degree of activation of i_{K2} and which follows first-order kinetics

$$ds/dt = \alpha_s(1-s) - \beta_s s \quad (2)$$

α_s and β_s are the rate constants which are voltage dependent only. The steady-state degree of activation of i_{K2} was measured in voltage clamp conditions^{2,4} as the amplitude of the current tail on the return to the holding potential. Since the holding potential is unchanged, $\bar{i}_{K2}$ must be the same and equation (1) can be modified

$$i_{K2} \propto s \quad (3)$$

Adrenaline shifts the S-shaped curve relating the steady-state degree of activation and the membrane potential (s_∞) as well as the reciprocal of the time constant ($\tau^{-1} = \alpha_s + \beta_s$) in the depolarising direction⁵⁻⁹. The instantaneous current-voltage relation,

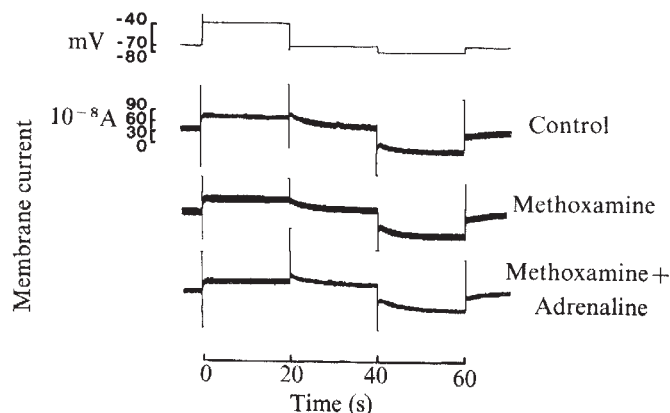


Fig. 1 Effect of methoxamine (4×10^{-5} g ml⁻¹) on voltage clamp currents in sheep Purkinje fibres. The Tyrode solution contained the following concentrations of ions (mM): Na⁺ 148.3, K⁺ 4, Ca²⁺ 1.8, Mg²⁺ 0.5, Cl⁻ 144.6, HCO₃⁻ 12, PO₄³⁻ 0.35, glucose 2 g l⁻¹. Each of the current records shows the response to 30 mV depolarisation and to 10 mV hyperpolarisation from a holding potential of -70 mV. Note that the time-dependent current (i_{K2}) is changed neither with methoxamine nor with methoxamine plus adrenaline. The instantaneous current jump following depolarisation vanishes with methoxamine and is restored by adding adrenaline in the presence of methoxamine.

however, is altered neither by adrenaline^{6,8}, nor by racemates⁸, nor optical isomers⁹ of various β -receptor blockers. It was previously thought that in heart muscle only β -adrenergic receptors are present and thus it was rather surprising when Cranefield *et al.*¹⁰ reported evidence for α -receptors in Purkinje fibres. Since agents specifically blocking β -adrenergic receptors shift the kinetics of i_{K2} back in the negative direction⁵⁻⁹ the question arose whether i_{K2} may also be influenced by drugs stimulating α receptors. Tsien⁶ found no detectable shift of the s -kinetics using phenylephrine (10^{-5} M) and propranolol (10^{-6} M), a well known β -receptor-blocking agent with strong local anaesthetic properties. Since in this (single) experiment two drugs were used—one of them (propranolol) with appreciable side-effects—it seemed appropriate to extend these studies and to use methoxamine (4×10^{-5} g ml⁻¹)¹⁰, a very selective α -receptor-stimulating agent with β -receptor-blocking activity¹¹ (provided by the Deutsche Wellcome GmbH).

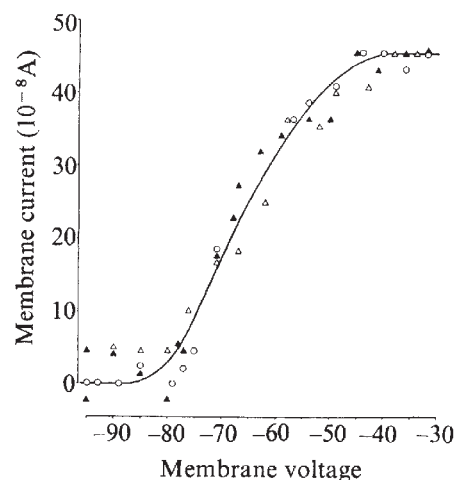


Fig. 2 Relationship between the steady-state degree of activation (s_∞) of i_{K2} and the membrane potential. $\circ$, Control; $\triangle$, methoxamine (4×10^{-5} g ml⁻¹); $\blacktriangle$, adrenaline (10^{-6} g ml⁻¹) plus methoxamine. Note that unlike the treatment with β -receptor-stimulating agents methoxamine as an α -adrenergic substance does not change the voltage dependence of s_∞ . Nor does adrenaline cause any shift in the presence of methoxamine.

In our experiments chart records of Purkinje fibre action potentials showed that a fibre was slightly depolarised with methoxamine. For an example in control conditions the resting potential amounted to -76 mV and decreased during the administration of methoxamine to -72 mV. Thus, it is not possible to tell whether the diminution of the amplitude of the action potential (and probably also of the rate of rise) observed after treatment with methoxamine was due to depolarisation only or whether this drug also reduces the availability of the excitatory sodium current at a particular membrane potential.

Weidmann's¹² h_∞ curve predicts a reduction in the rate of rise by about 25% due to a depolarisation of the order we observed. But in spite of the slight depolarisation, the time course of the pacemaker potential was essentially unchanged.

Figure 1 shows the membrane current changes as responses to long-lasting depolarising and hyperpolarising clamp pulses. There was no measurable change in the time-dependent currents. The only alteration was in the non-time-dependent background current called i_{K1} (refs 2 and 13). This instantaneous current changed in the inward direction—that is, the outward current jump after depolarisation became negligible when methoxamine was present; during and after hyperpolarisation an increase in i_{K1} could hardly be detected. But a decrease in an instantaneous outward current would be expected to depolarise the fibre. This current change was reversed when adrenaline was added together with methoxamine.

Figure 2 shows the relationship between the steady-state

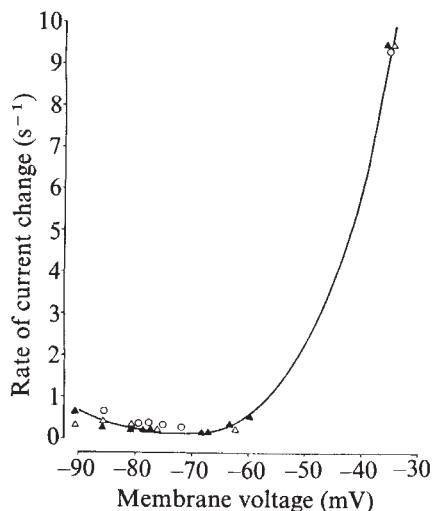


Fig. 3 Voltage dependence of the rate of change of i_{K2} measured in terms of reciprocal of the time constant (τ^{-1}) of current change. Symbols as in Fig. 2.

degree of activation of i_{K2} and the membrane potential measured as the amplitude of the current tail on return from various clamp potentials to the holding potential. This curve was shifted neither with methoxamine nor with methoxamine plus adrenaline (Fig. 2).

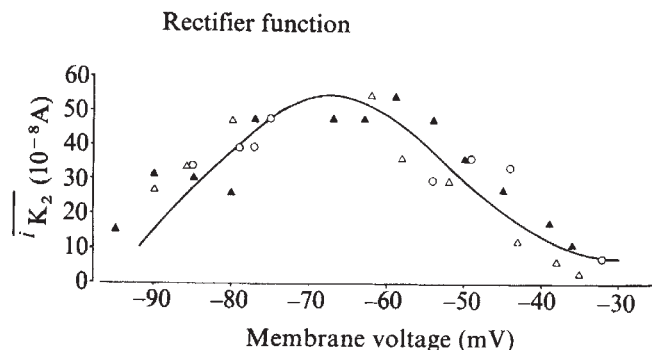
The absence of any shift in s_{∞} by adrenaline in the presence of methoxamine was probably a consequence of the β -receptor-blocking activity of this drug¹¹.

The time constant of i_{K2} at potentials less negative than the pacemaker region of potentials is determined by a procedure called the 'envelope test'² in which the duration of the clamp to a particular potential is progressively shortened, thereby switching on i_{K2} to various degrees.

The time constant of i_{K2} was identical at a particular potential whether determined in control conditions, in the presence of methoxamine or in the presence of methoxamine plus adrenaline. τ_s was measured as the amplitude of current tails on return to the holding potential following clamps to -36 mV of various durations and was found to be 106 ms. Nor is there any shift in the U-shaped curve relating the reciprocal of the time constant and the membrane potential (Fig. 3).

The kinetics of i_{K2} —or the second factor in equation (1)—are related to the electric field at the membrane and thus the voltage dependence of the kinetics may be modified by altering the amount of electric charges at the inner or outer edge of the

Fig. 4 Instantaneous current-voltage relationship of i_{K2} . This relationship is obtained from the quotient of the amplitudes of i_{K2} during and following voltage clamp pulses to various levels. The 'rectifier ratio' obtained in this way is then multiplied by the amplitude of s_{∞} measured at the same holding potential, resulting in the value of the 'rectifier function', $\bar{i}_{K2}$. The absolute magnitude of i_{K2} is altered neither with methoxamine nor with methoxamine plus adrenaline and the rectifier properties are unchanged with either condition. Symbols as in Figs 2 and 3.



membrane. The findings described here show that stimulation of α -receptors does not influence the electric field near the i_{K2} channel.

The instantaneous current voltage relation of i_{K2} usually shows a marked inwardly directed rectification and a considerable negative slope².

The results reported here (Fig. 4) demonstrate that neither methoxamine nor methoxamine plus adrenaline alter the absolute magnitude or the rectifier properties of i_{K2} .

Our results support the idea that i_{K2} is governed by β -receptors⁶⁻⁹ and they exclude the possibility that α -receptors influence i_{K2} . Thus, it is not surprising that drugs selectively stimulating α -receptors do not have a positive chronotropic action on cardiac muscle.

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Spontaneous repetitive hyperpolarisations from cells in the rat adenohypophysis

SPONTANEOUS, repetitive potential changes in the form of action potentials have been observed in only a few types of highly specialised cells such as cardiac pacemaker cells, various smooth muscle cells and certain nerve cells. Some endocrine cells, however, can exhibit repetitive depolarisations with similarities to action potentials; for example, the beta cells of the pancreas¹, cells in the adrenal cortex² and cells of a clonal line of an anterior pituitary tumour³. We report here that some cells in the adenohypophysis of ovariectomised rats are electrically excitable as well as exhibiting spontaneous, repetitive hyperpolarisations.

The rats were ovariectomised 3–5 weeks before the experiments, to induce hypertrophy of the pituitary gonadotropic cells and produce a more uniform population of cells from which more stable recordings of membrane potentials could be obtained. One hemilobe of the anterior pituitary gland was mounted in a Perspex chamber and superfused with Krebs–Henseleit bicarbonate solution at 37 °C, in a manner similar to that described before¹. Membrane potentials were recorded from superficial cells by means of glass microelectrodes filled with 5 M potassium acetate with a resistance of 70–150 M Ω . A bridge circuit was used for intracellular recording and injection of current.

It was difficult to obtain stable measurements of membrane potentials from the cells of the adenohypophysis, but in 13

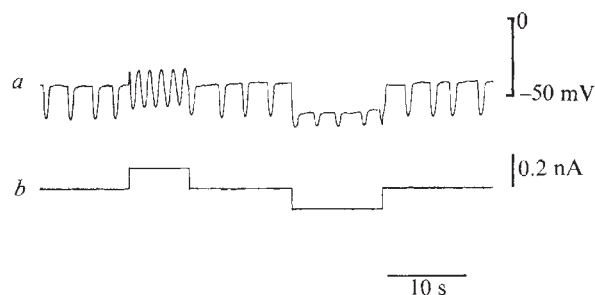
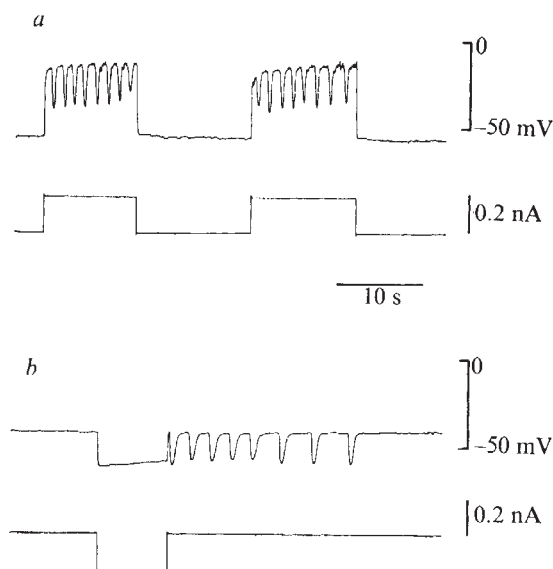


Fig. 1 Intracellular recording from an anterior pituitary cell exhibiting spontaneous, repetitive hyperpolarisation. The cell, which had a resting membrane potential of -42 mV, exhibited spontaneous hyperpolarisations at a frequency of 25 min^{-1} for 4 min before the record in *a* was obtained. The left part shows the effect of a depolarising current, and the right shows the effect of a hyperpolarising current. *b* Indicates that the current passed through the microelectrode. This and all subsequent records, were made by means of a pen recorder and the pituitary was obtained from a rat ovariectomised 4 weeks before study.

successful experiments recording from 127 cells the mean value was -49.3 ± 2.6 mV (s.e. of mean), which is more negative than previous *in vivo* measurements⁴ (-21 mV) and *in vitro* measurements on pituitary tumour cells³ (-41 mV). In eight experiments in which the input resistance—taken as a measure of the cell membrane resistance—was determined by passing small depolarising currents with the bridge balanced before and after penetration, the mean value was $118 \text{ M}\Omega$ (range $5\text{--}330 \text{ M}\Omega$). This value is slightly larger than that found in a previous study⁴ of *in vivo* rat pituitary ($42 \text{ M}\Omega$) but less than the value observed³ with a clonal line of pituitary tumour cells ($340\text{--}700 \text{ M}\Omega$).

One population of cells with a mean resting potential of -37.1 ± 1.7 mV ($n=12$) and a mean resistance of $111 \pm 31 \text{ M}\Omega$ ($n=7$) was peculiar in exhibiting spontaneous, repetitive hyperpolarisations. The mean frequency was 37.5 min^{-1} (range $2\text{--}65$) and the mean amplitude was 16.0 mV (range $8\text{--}26$). The record shown in Fig. 1 demonstrates the phenomenon and shows that the frequency of the hyperpolarisations increased when a small depolarising current was passed through the membrane. When the cell was hyperpolarised by a current passed in the opposite direction, the amplitude of the hyperpolarisations was reduced substantially, whereas the frequency

Fig. 2 Intracellular recordings demonstrating the excitability of the hyperpolarising cells. *a*, Ability of a depolarising current (indicated on the lower trace) and *b*, ability of termination of a hyperpolarising current (indicated on the lower trace) to evoke repetitive hyperpolarisations from an otherwise inactive cell.



did not change appreciably. Although most of these cells continued their repetitive hyperpolarisations for as long as 24 min, in a few cases the hyperpolarisations ceased even though the resting potential remained unchanged. In these cells, as well as in some cells which did not exhibit spontaneous hyperpolarisations, it was possible to initiate repetitive hyperpolarisations either by passing a depolarising current or by termination of a hyperpolarising current (Fig. 2).

A few cells transiently increased their resting potentials to values as negative as -75 mV. When this happened, the magnitude of the repetitive hyperpolarisations decreased successively and they were abolished at membrane potentials ranging from -55 to -70 mV. When the resting potential exceeded the value at which the hyperpolarisations were abolished, the polarity of the potential changes was reversed. Subsequently the resting potential of these cells returned to the original value (close to -40 mV) whereby the hyperpolarising behaviour was restored. It was also possible to change the spontaneous, repetitive hyperpolarisations to depolarisations by passing a hyperpolarising current sufficient to increase the resting potential beyond the 'reversal potential' (Fig. 3*a*).

Since hyperpolarisation of cell membranes is frequently a result of an increased permeability to potassium ions, we

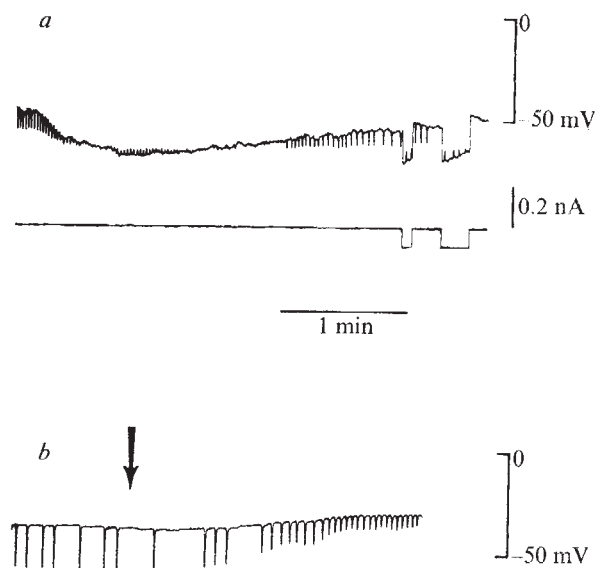


Fig. 3 Intracellular recordings from spontaneously, repetitively hyperpolarising cells. *a*, Changes in amplitude and polarity of the spontaneous, repetitive potential changes caused by variations in the resting membrane potential. The left part shows the effect of a spontaneous increase in the resting potential, and the right shows the effect of hyperpolarisation caused by a current (indicated on the lower trace) passed through the microelectrode. *b*, Effect of increasing the extracellular potassium ion concentration on amplitude and frequency of repetitive hyperpolarisations. From the time indicated by the arrow, a modified Krebs-Henseleit bicarbonate solution with a potassium ion concentration of 47 mM (10 times normal, sodium ion concentration reduced correspondingly) was passed through the superfusion chamber at a rate of 0.78 chamber volume per min.

studied the effect of raising the potassium ion concentration of the superfusion fluid tenfold (to 47 mM). Figure 3*b* demonstrates the resulting decrease in the amplitude of the hyperpolarisations; the hyperpolarisations were subsequently abolished (not shown). The resting membrane potential was reduced by the high potassium ion concentrations, and this reduction was associated with an increase in the frequency of the hyperpolarisations. All the effects of the high extracellular potassium ion concentration were reversible.

Although it is not known from which cells in the adenohypophysis the spontaneous, repetitive hyperpolarisations originate, it is possible that the potentials have been recorded from

the gonadotropic cells, which exhibit selective hypertrophy after ovariectomy. Our inability to record spontaneous, repetitive hyperpolarisations from the adenohypophyses of normal rats gives some support to this view.

In cells able to hyperpolarise in response to stimulation, for example, nerve cells stimulated by inhibitory transmitter substances⁵, salivary gland acinar cells stimulated by autonomic nerves⁶ and certain photoreceptor cells stimulated by light⁷, a common underlying mechanism is a stimulation-induced increase of the conductance of the cell membranes to potassium ions. In contrast, the cells in the adenohypophysis can produce repetitive hyperpolarisations in the absence of any obvious discontinuous, repetitive stimulus. The effect of increasing the extracellular potassium ion concentration indicates that a cyclic, transient increase in the conductance of the cell membrane to this ion may be involved in the hyperpolarisations. On the other hand, the value of the reversal potential is compatible with additional conductance changes to other ions such as sodium and calcium. The latter possibility is interesting because uptake of extracellular calcium ions is an important step in many secretory processes⁸.

Although our results suggest that the hyperpolarising cells have pacemaker-like properties, there is an alternative possibility. If the hyperpolarising cells were connected to spontaneously discharging nerve cells through both an inhibitory chemical and an electrical synapse, the observed behaviour could be explained by postulating that the electrical synapse showed rectification. Neurones connected by both electrical and chemical synapses have been demonstrated^{9,10}; neurones innervating adenohypophyseal secretory cells, however, have not been demonstrated.

The excitability of the hyperpolarising cells (Figs 1 and 2) show certain similarities to the excitability found in depolarising pituitary tumour cells by Kidokoro³. The cells described here however, are unique both in hyperpolarising in response to direct electrical stimulation and in showing repetitive hyperpolarisations.

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Increase in microviscosity with ageing in protoplast plasmalemma of rose petals

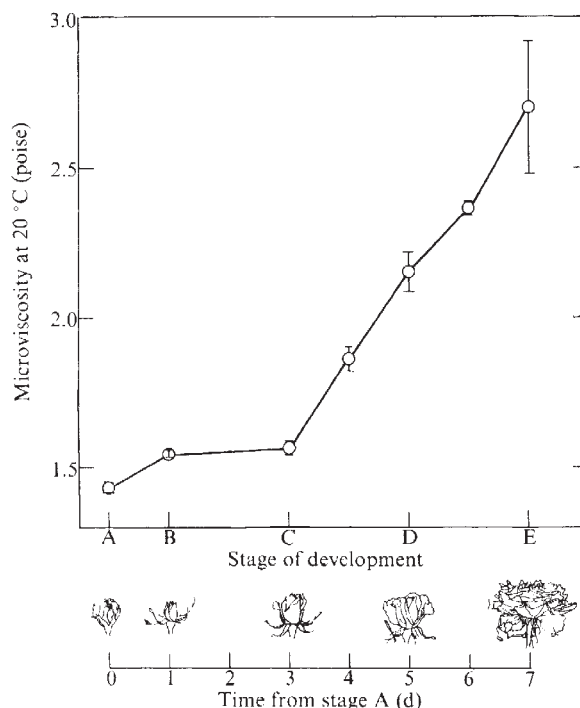
It is now widely accepted that most physiological functions of biological membranes are related to the dynamic characteristics of transport, enzyme and receptor sites. Since the rotational and translational mobility¹, as well as the degree of exposure of membrane proteins², are largely determined by the fluidity of the lipid layer, membrane microviscosity may be important

in regulating various physiological activities, as in lymphocytes^{3–5} and erythrocytes⁶. To our knowledge, changes in fluidity of plant plasmalemma, the outer surface of the protoplast⁷, have not yet been reported. We report here a gradual increase in microviscosity of protoplast plasmalemma from petals of ageing rose flowers.

Rose (*Rosa* hyb.) flowers cv. Golden Wave (Syn. Dr. Verhage) were allowed to develop and age on the plant and were picked at different stages of development—from a tight bud to a fully opened flower with first signs of wilt and colour fading. The four outer petals of the flowers were macerated enzymatically in 0.6 M mannitol (Merck) containing 0.5% cellulysin (Calbiochem) and 0.25% driselase (Kyowa Hakko Kogyo Co.) by incubation for 18 h at 20 °C in the dark. As a reporter molecule, we used 1,6-diphenyl-1,3,5-hexatriene (DPH), the most efficient probe available for monitoring fluidity properties of lipid regions by fluorescence polarisation^{2–4,8}. For labelling, isolated protoplasts were incubated for 1 h at 37 °C with 10^{–6} M dispersion of DPH in 0.6 M mannitol. The labelled protoplasts were then washed and resuspended in 0.6 M mannitol to a concentration of 5 × 10⁶–8 × 10⁶ ml^{–1}. A fluorescence microscopic view of a DPH-labelled protoplast is of a glowing periphery, indicating that the fluorescence signal is generated predominantly from the hydrocarbon layer of the protoplast plasmalemma.

Fluorescence polarisation and intensity in the labelled protoplast suspension were recorded, and the corresponding microviscosity evaluated by a method described previously^{2,3,8}. Figure 1 shows the change in microviscosity of the protoplast plasmalemma with stage of development of the petals. As shown, the membrane microviscosity ($\bar{\eta}$) gradually increases with the flower development. The rate of increase in $\bar{\eta}$ is markedly enhanced during the 4-d period between stage C (partially opened flower) and stage E (wilting flower), when the microviscosity is approximately doubled. The effect of temperature on the plasmalemma microviscosity was also determined in each stage in the temperature range of 4–30 °C. For all membranes, the change of log $\bar{\eta}$ with the reciprocal of the absolute temperature (1/T) followed a straight line with an apparent flow activation energy (ΔE) of 3.4 ± 0.2 kcalorie mol^{–1}. As an

Fig. 1 Microviscosity of protoplast plasmalemmas obtained from rose petals at different stages of development. Bars represent the range of values obtained with protoplasts from 3–5 different flowers.



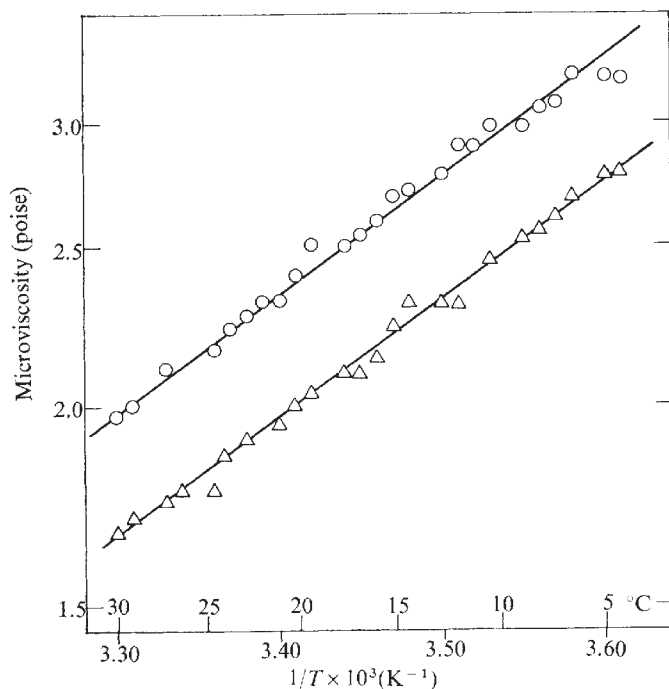


Fig. 2 Temperature dependence of microviscosity in protoplast plasmalemmas from rose petals at stages D (Δ) and E ($\circ$). Each point represents an average of three values obtained with different preparations.

example, Fig. 2 shows plots of $\log \bar{\eta}$ against $1/T$ obtained for plasmalemma from stages D and E (see Fig. 1).

The data now available for plant membranes are still few and insufficient. The conclusions that can be drawn from this study are therefore bound to facts which were established for mammalian membranes. The unusually low and invariable value of $\Delta E = 3.4$ kcalorie mol^{-1} (in mammalian membranes $\Delta E = 6.5\text{--}8.5$ kcalorie mol^{-1} , (see ref. 2)) indicates that the lipid domains of the plasmalemma are highly ordered, even though they maintain a relatively high fluidity. In addition, it suggests that the membrane function and organisation are only slightly affected by temperature changes, which may be a special feature of plant membranes. A combination of these properties has no parallel in mammalian membranes, and indicates that the lipid composition, organisation and dynamics of plant and mammalian membranes are basically different. Nevertheless, the increase of plasmalemma microviscosity with the age of the flower could result primarily from one (or a combination) of the following changes: (1) an increase in the molar ratio of sterols to phospholipids; (2) a decrease in the degree of unsaturation of the fatty acid chains; (3) an increase in the relative amount of membrane proteins. Changes of the first⁹ and the second¹⁰ kind have been observed in aged plant tissues. The increase in microviscosity caused by such changes may decrease the turnover of membrane enzymes and hormone receptors and thus attenuate some metabolic processes which will eventually lead to the death of the flower¹¹⁻¹³.

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Spin label study of erythrocyte membrane fluidity in myotonic and Duchenne muscular dystrophy and congenital myotonia

INTACT erythrocyte membranes from patients with myotonic muscular dystrophy (MMD) have been shown by spin labelling (review of method in ref. 1) to have greater membrane surface fluidity than normal^{2,3}. We have now evaluated the specificity of this phenomenon. We used erythrocytes from patients with MMD, and also from patients with Duchenne muscular dystrophy (DMD), as a model of dystrophy with no myotonia, and from patients with congenital myotonia (CM) as a model of myotonia without dystrophy.

MMD has been considered a defect of muscle surface membrane based on the persistence of repetitive membrane depolarisation after nerve block or neuromuscular blockade⁴. A similar locus has been postulated for the membrane defect in congenital myotonia⁴. Our previous biochemical and biophysical studies have suggested that in MMD, there are membrane abnormalities in erythrocytes as well as muscle membranes^{2,3,5-8}. Membrane protein phosphorylation is lower than normal^{5,6} and the stoichiometry of the sodium pump is unusual⁷. An abnormally large number of stomatocytes was noted by scanning electron microscopy in MMD as well as several other inherited muscle diseases, including CM and DMD⁸. Studies with stearic acid methyl ester spin labels have demonstrated that MMD erythrocyte membranes are more fluid near the membrane surface than are those of normal controls^{2,3}. The data now reported indicate similarly high erythrocyte fluidity near the membrane surface in CM, whereas erythrocytes from DMD and other non-myotonic muscular dystrophic patients have normal membrane fluidity as assessed using 5-NMS (see below). These results suggest a correlation of increased membrane fluidity with the presence of myotonia.

The principal spin label we used was 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinylmethyl ester (5-nitroxide methyl stearate) (5-NMS) (Syva Associates). The erythrocytes were obtained from fresh heparinised blood from patients and from age and sex-matched controls. In experiments with MMD cells, data previously obtained³ from seven normal and nine myotonic patients were recalculated as described below. Samples from twelve normal and ten DMD patients and nine normal and nine CM patients, respectively, were used. In each of the three separate sets of experiments, intact erythrocytes were prepared by centrifuging blood at 1,570g for 10 min at 4 °C and washing the cells four times with 5 mM sodium phosphate-150 mM NaCl buffer, pH 8.0 (PBS). The buffy coat was removed carefully and the cells were resuspended in isotonic PBS at a haematocrit of 45-50%.

Intact MMD cells were labelled overnight at 37 °C and intact CM and DMD cells were incubated with 5-NMS at room temperature for 10-30 min. In each respective set of experiments, control and disease state membranes were treated identically. The ratio of spin label to lipid molecules were kept at approximately 1:50 to avoid spin exchange effects⁹. Magnetic resonance measurements were performed as before³ on both a Varian V-4502-15 and a Ventron-Magnion MVR-9X electron spin resonance (ESR) spectrometer, with a quartz aqueous sample cell.

The spin label we used is thought to orient in the erythrocyte membrane with the polar head group near the lipid head group and the alkyl chain parallel on the average to the lipid alkyl

chains. Rapid anisotropic rotation takes place about the long axis of the spin label whose nitroxide group probes an environment near the membrane surface. A typical spectrum of 5-NMS in erythrocyte membranes is shown in Fig. 1, in which the experimental T -tensor parameters are indicated. The order parameter, S , is a measure of the fluidity of the local environment in which the paramagnetic centre of the spin label is found while a_N , the nitrogen isotropic hyperfine coupling constant, is a probe of local polarity. Regardless of whether the random walk model of Jost *et al.*¹⁰ or the model of Mason *et al.*¹¹ (which involves an inverse relationship between the rate of rotation of the symmetry axis and the apparent values of both S and a_N) is used to explain spectra like those of Fig. 1, the interpretation of S as a measure of local fluidity is valid. The polarity correction factor used by Hubbell and McConnell¹² with the crystal T -tensor values of "doxyl"-propane reported by Jost *et al.*¹⁰, were used in the calculation of S .

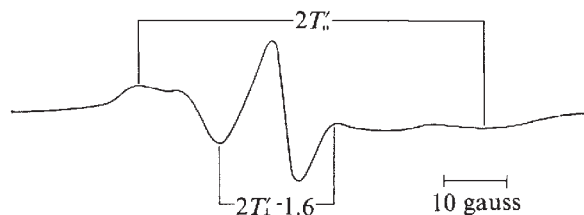


Fig. 1 Typical spectrum of 5-NMS in normal erythrocytes. The tensor parameters are indicated.

The mean S values for controls and each disease state performed in the three separate sets of experiments were compared by a two-way analysis of variance¹³ (Table 1). This two-tailed method of statistical analysis minimises possible fluctuations from day to day between separate experimental values which can often occur with biological samples.

P is the significance of the difference of the mean values of S_{control} and $S_{\text{disease state}}$ (or $(a_N)_{\text{control}}$ and $(a_N)_{\text{disease state}}$) in each set of experiments calculated using a two-way analysis of variance. The null hypothesis was that MMD, DMD and CM membranes are, respectively, not different from the corresponding controls. The difference of S_{control} and S_{MMD} or S_{CM} is significantly greater than zero, indicating more fluid environments in erythrocytes from these myotonic conditions. In contrast, there is no mean difference of S_{control} and S_{DMD} and, accordingly, no difference in surface membrane fluidity. In this latter case the data actually tended to show DMD erythrocytes to be more rigid than controls but statistical significance of this observation could not be demonstrated.

The difference between the mean values of the control parameters in the MMD experiments compared with those in the CM and DMD experiments probably reflects the different

methods of labelling used. Intact erythrocytes held for several hours at 37 °C rapidly lose intracellular ATP with a consequent decrease in cell deformability¹⁴. This latter property may reflect an increased membrane rigidity which in turn would demonstrate an increased order parameter. Intact erythrocytes labelled overnight at 37 °C show an increased S -value when compared with cells from the same donor labelled for 15 min at room temperature (D.A.B., unpublished). The a_N value of 5-NMS in control cells is also increased in the MMD experiments compared with those of the other two sets of experiments (Table 2), suggesting that the spin probe is experiencing a different environment in the former case. The important aspect of these data is that within each separate set of experiments, normal and disease state membranes were treated in the same manner.

Our previous results³ recalculated by the two-way analysis of variance show MMD erythrocyte membranes to have greater than normal surface fluidity as assessed by 5-NMS and less than normal local polarity, as measured by 16-NMS, relative to those of normal controls. The study reported here demonstrates that membrane fluidity is greater than normal in CM but not different from controls in DMD, hyperkalaemic periodic paralysis (without para-myotonia) or oculopharyngeal muscular dystrophy. There are no highly significant polarity changes as measured by a_N in the various experiments (Table 2).

We also found greater than normal membrane fluidity in MMD and CM erythrocytes when we used the corresponding acid spin label (5-NS) in identical procedures. The same differences of fluidity in erythrocyte ghost preparations⁶ as in intact cells were also observed.

Table 2 Comparison of a_N for 5-NMS in normal and MMD, DMD or CM intact erythrocytes

	N	MMD	N	DMD	N	CM
Mean	15.91	15.81	15.56	15.67	15.50	15.43
s.e.m.	0.07	0.05	0.05	0.31	0.31	0.32
n	7	7	10	10	9	9
P	<0.25		<0.25		<0.25	

The data for MMD were from ref. 3, recalculated by a two-way analysis of variance.

P was calculated as in Table 1.

Increased membrane fluidity has been demonstrated in both MMD and CM erythrocyte membranes by means of ESR spectroscopy. Although this method detects a fluidity increase in both cases, the specific biochemical alterations responsible for the change in these diseases may be quite different. Lower than normal phosphorylation of component a has been demonstrated in MMD erythrocyte ghosts, while CM ghosts do not have such biochemical alteration¹⁶. Physiological data also support different mechanisms of myotonia. Muscle membranes from CM demonstrate greater than normal resistance and less than normal chloride conductance, while these characteristics are not apparently affected in MMD⁴. It is interesting that the two myotonic diseases MMD and CM involve abnormal membrane fluidity of erythrocytes whereas DMD and the other two non-myotonic dystrophies apparently do not.

Membrane fluidity is a function of many variables, including the nature of membrane lipids (type of phospholipid, fatty acid chain length and degree of unsaturation, head group and cholesterol content), temperature, water content, presence of ions (especially divalent cations), and the total of protein-lipid interactions^{16,17}. Activity of membrane-associated transport enzymes in bacterial¹⁸ and animal^{19,20} systems can be altered by affecting membrane fluidity. Increased membrane fluidity in MMD and CM may affect the expression of a transport or enzyme system resulting in the clinical sign of myotonia in muscle. Although the specific mechanism responsible for these

Table 1 Comparison of the order parameter S for 5-NMS in normal and MMD, DMD or CM intact erythrocytes

	S_N	S_{MMD}	S_N	S_{DMD}	S_N	S_{CM}
Mean	0.625	0.590	0.598	0.605	0.588	0.570
s.e.m.	0.008	0.006	0.012	0.015	0.010	0.008
n	7	9	12	10	9	9
P	<0.005		0.1 < P < 0.25		<0.01	

N, normal. S is calculated by

$$\frac{T_{11}' - T_{11}}{T_{11} - T_{11}} \frac{a_{x1}}{a'}$$

where the primed values are obtained experimentally (Fig. 1) and $a' = (T_{11}' + 2T_{11}')/3$. The unprimed crystal values are obtained from the results of Jost *et al.*¹⁰ on doxyl-propane. The data for MMD were from ref. 3, recalculated by a two-way analysis of variance. Data from three separate sets of experiments are presented. P was calculated by a two-way analysis of variance using a two-tailed test with a null hypothesis that the respective means are equal.

phenomena is unknown, our spin label measurements suggest a correlation of increased erythrocyte membrane fluidity with the presence of myotonia.

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Vitamin D-stimulated intestinal calcium absorption may not involve calcium-binding protein directly

ONE of the earliest responses of chick intestine to cholecalciferol (vitamin D₃) is the appearance of a soluble calcium-binding protein (CaBP) for which current evidence indicates a role in the intestinal absorption of calcium^{1,2}. CaBP, which has never been detected in the intestine of cholecalciferol-deficient chickens, is synthesised *de novo* in response to cholecalciferol, apparently by the induction of the transcription of a specific mRNA for CaBP (ref. 3). In the chick CaBP biosynthesis is initiated after a lag of about 7 h after physiological doses of the vitamin. A correlation between the appearance of CaBP and the first detectable increase in calcium absorption in response to cholecalciferol was observed when CaBP was measured using immunological procedures^{4,5} but not using the less sensitive Chelex assay⁶. Consequently it has been generally accepted that CaBP plays a major role in chicks in cholecalciferol-stimulated calcium transport, but by a mechanism which has still to be described. These changes in intestinal

CaBP levels and in calcium absorption are brought about by 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃), the hormonal metabolite of D₃. We report here the sequence and time scale of changes in calcium absorption and CaBP levels in the intestine in response to 1,25-(OH)₂D₃. By comparison with events observed after cholecalciferol administration, the changes in response to the hormone turn out to be much more rapid and to have different rates of decay, with calcium transport being stimulated before CaBP can be detected immunologically.

Using immunoelectrophoresis⁷ we have measured the amount of CaBP in the intestinal mucosa of rachitic chicks at various intervals after injecting 1,25-(OH)₂D₃ intracardially. CaBP was first detected 5 h after dosing (7 h earlier than after an equivalent dose of cholecalciferol) and by 16 h had increased to a level of 35 µg per g wet weight mucosa. Maximal quantities (42 µg g⁻¹) were obtained by 48 h, the levels declining to about 50% of the maximum by 72 h (Table 1); the protein could not be detected again by 96 h.

The ability of rachitic chicks to transport calcium across the intestine after receiving 1,25-(OH)₂D₃ was measured *in vitro* using everted intestinal sacs⁸. Sacs prepared from birds killed

Table 1 Calcium-binding protein levels in intestinal mucosa of rachitic chicks treated with 1,25-(OH)₂D₃

Time (h) after dosing	CaBP (µg per g wet weight tissue)	% Maximum response
0	0	0
5	*	
7	12.0	28.5
8	22.5	53.6
16	35.0	83.3
24	36.3	86.4
48	42.0	100
72	20.0	47.6
96	0	0

* CaBP was detectable but in amounts too low for measurement.

Groups of six rachitic chicks received intracardial injections of 125 ng 1,25-(OH)₂D₃ in 0.1 ml 10% EtOH and 90% propylene glycol, and were killed at the times shown. CaBP was measured in the pooled duodenal cytosols by immunoelectrophoresis⁷ using pure CaBP as standard.

2 h after dosing, transported calcium at a rate significantly higher than that of the controls, the maximum rate being reached by sacs obtained from birds 8 h after dosing (Fig. 1). By 21 h the ability of the sacs to transport calcium had declined to a rate slower than that occurring at 2 h. Thus by 4 h, 1,25-(OH)₂D₃-stimulated calcium transport was occurring at more than 50% of the maximum measured rate, whereas CaBP could not be detected. By 21 h, when calcium transport had declined markedly, the intestinal CaBP level was approaching its maximum. This lack of correlation between calcium transport and the quantity of CaBP induced by a single injection of 1,25-(OH)₂D₃, at both short and longer time intervals, suggested that the production of CaBP might be an event secondary to the hormonal stimulation of calcium transport. We therefore re-examined the phenomena using more sensitive methods for the detection of CaBP.

In the chick, cholecalciferol-stimulated CaBP appears to the greatest extent in the duodenum⁹. We therefore tested the ability of duodenal polysomes prepared from rachitic and 1,25-(OH)₂D₃-dosed chicks to synthesise CaBP in an *in vitro* cell-free system¹¹. Incorporation of ³H-leucine into completed polypeptide chains and into immunoprecipitable CaBP was determined. The specificity of the antiserum used to precipitate the tritiated CaBP was confirmed by analysing the dissociated immunoprecipitates on polyacrylamide gels. Typically, more than 80% of the total radioactivity recovered from the gel slices was localised in the CaBP peak. To measure CaBP synthesis, radioactivity in the CaBP area of the gels was related to the total TCA-precipitable incorporation into completed polypeptide chains.

Table 2 Effect of 1,25-(OH)₂D₃ on the synthesis of CaBP by polysomes *in vitro*

Time (h) after dosing	Radioactivity in immunoprecipitable CaBP (d.p.m.)	CaBP (% total proteins synthesised by polysomes)	% Maximum response
0	0	0	0
1*	0	0	0
2*	517	0.04	1.10
3	1,091	0.08	2.33
4	9,774	0.69	20.1
6	27,242	1.92	58.2
8	37,790	2.66	80.8
13	46,788	3.29	100
21	8,170	0.57	17.5
48	0	0	0

*Same birds were used for both polysome preparation and calcium transport measurements. At each other time, birds were taken from the same batch as those used for calcium transport.

Groups of four rachitic chicks received 125 ng 1,25-(OH)₂D₃ intracardially and were killed at the indicated times. Duodenal polysomes were prepared¹⁰ and incubated for 40 min at 37 °C in triplicate 330-μl aliquots. The incubation medium contained 1 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, 40 μg ml⁻¹ creatine kinase, 50 μM of each amino acid except leucine, 5 μCi ml⁻¹ ³H-leucine, 0.5 mg ml⁻¹ polysomal RNA, 0.4 ml ml⁻¹ gel-filtered rat liver cell sap, 1 mM dithiothreitol, 4 mM MgCl₂, 100 mM KCl and 20 mM Tris-Cl pH 7.5. Duplicate 20 μl aliquots of the pooled triplicate postribosomal supernatants were removed for measurement of TCA-precipitable radioactivity in released protein. The remainder was immunoprecipitated with anti-CaBP as described previously³. The immunoprecipitate was dissolved by heating for 20 s at 90 °C in 100 μl SDS-Tris-dithiothreitol solution¹² and electrophoresed on SDS-acrylamide gels, which were then cut into 1-mm slices and processed for radioactive counting³. ³H radioactivity (d.p.m.) in the CaBP area of the gels is expressed as a percentage of the total TCA-precipitable incorporation into released proteins (corrected up to the maximum value obtained, that is, 1.4 × 10⁶ d.p.m. ml⁻¹) at each interval.

As shown in Table 2, CaBP mRNA activity was maximal 13 h after a single hormone injection, by which time CaBP represented 3–4% of the total protein-synthesising capacity of the polysomes (assuming a constant rate of translation). Polysomes from birds killed 1 h after receiving 1,25-(OH)₂D₃ were unable to synthesise CaBP. At this time, however, intestinal sacs prepared from the same birds could transport calcium at a low level (Fig. 1). Sacs prepared from birds killed 2 h after dosing, transported calcium at 27% of the maximum rate, but polysomes from these birds could only synthesise minute amounts of CaBP equivalent to 0.04% of the total proteins completed *in vitro*. Therefore, although translation

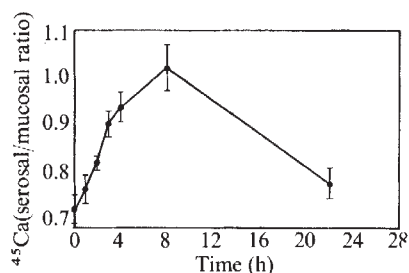


Fig. 1 Stimulation of *in vitro* calcium transport in chick intestine in response to 1,25-(OH)₂D₃. Rachitic chicks (other than controls) were dosed intracardially with 125 ng 1,25-(OH)₂D₃ and killed at the times shown. Sacs were constructed from 3.0 cm of ileum distal to the yolk sac remnant and filled with 0.3 ml medium. Incubation was carried out at 37 °C for 90 min with shaking in 25-ml Erlenmeyer flasks containing 3.0 ml of the same medium in 95% O₂/5% CO₂. A low sodium medium was used to reduce water transport¹¹; this contained 198 mM mannitol, 25 mM KCl, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 1.2 mM MgSO₄, 20 mM glucose, 0.5 mM CaCl₂ and ⁴⁵Ca at a concentration of 40,000 c.p.m. ml⁻¹. Aliquots (100 μl) were removed from the serosal and mucosal solutions after incubation for measurement of radioactivity. Each point is the mean of a group of 7–10 chicks. Vertical bars indicate ± s.e.m. A low level of calcium transport seemed to be taking place after 1 h (*P* < 0.3) but significant transport did not occur until 2 h (*P* < 0.001).

of CaBP mRNA *in vivo* had commenced by 2 h after dosing, CaBP production represented only 1.0% of the maximum activity attained.

Thus, at all times at which calcium transport could be observed at a significantly higher rate in dosed chicks than in rachitic birds, we detected nascent CaBP on the polysomes. We have also detected very low amounts (0.01% of total protein synthesis) of soluble (that is, completed, released from polysomes and presumably functional) ³H-CaBP synthesised *in vitro* by intestinal slices obtained from birds killed 2 h after

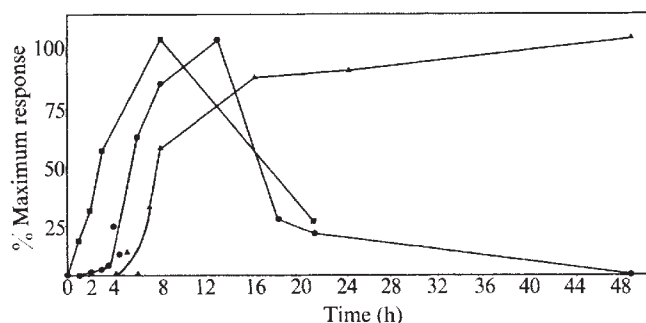


Fig. 2 Time course of events occurring in the rachitic chick intestine after a single intracardial injection of 125 ng 1,25-(OH)₂D₃. ■, Calcium transport *in vitro*; ●, CaBP mRNA activity; ▲, CaBP level in intestinal cytosol.

dosing (data not shown), confirming the presence of minute amounts of CaBP in the intestine at this stage. None was detectable at 1 h. From the composite data presented in Fig. 2, however, by 3 h after dosing, when calcium transport is proceeding at 50% of its maximum rate, CaBP biosynthesis is occurring at only 2.5% of its maximum rate, and in general the response curve of the latter lies about 2.5 h after the former.

It is also apparent from Table 2 and Fig. 2 that after 13 h the ability of the polysomes to synthesise CaBP declined rapidly, being completely absent by 48 h. This suggests that the half life of CaBP mRNA is not more than 4 h; high levels of CaBP observed in intestinal supernatants between 16 and 48 h and its disappearance between 48 and 96 h therefore indicate that the accumulated CaBP is stable within the 3–4-d life of the cell, rather than that a steady-state situation of continued synthesis and breakdown exists. In addition, the continued presence of CaBP in mucosal cells at times when calcium transport is no longer detectable indicates that other vitamin D-dependent factors are required.

There have been reports of a correlation between changes in calcium absorption and in CaBP levels which occur in chicks in response to cholecalciferol^{4–6}. Before the present study, it seemed that a correlation could be demonstrated providing that the CaBP levels were measured by immunological procedures. Furthermore, the available information from two laboratories^{13,14} on the effect of 1,25-(OH)₂D₃ on chick intestine seemed to support this view, but in both studies the times at which comparisons were made were too late to detect calcium transport in the absence of CaBP. In our studies, in which changes in calcium absorption and CaBP after 1,25-(OH)₂D₃ were compared at hourly intervals, calcium transport occurred while CaBP was absent or present in very small amounts. Thus, it is unlikely that CaBP is required for the initial calcium transport occurring in response to 1,25-(OH)₂D₃, although the possibility remains that only a small proportion (0.01–1.0%) of the CaBP ultimately synthesised in response to the hormone actively promotes calcium transport. If such a small proportion of CaBP is active in calcium absorption there are implications for the mechanism by which the protein is effective. That finding would imply a catalytic role for CaBP rather than its functioning in a stoichiometric relationship with the calcium being transported¹⁵. In addition, the intracellular distribution of this small quantity may be different from the distribution of the total CaBP eventually produced by the cell¹⁶. It seems

unlikely, however, that the bulk (> 99%) of the CaBP in the mucosal cell is inactive. We therefore conclude that there is not an obligatory requirement for CaBP in all calcium transport.

Whether such a conclusion is applicable to mammalian intestine awaits further investigation. The present position is that the absence of CaBP in the intestine of rachitic animals of some species as claimed by some investigators^{17,18} is open to doubt^{19,20}, although there is general acceptance that its synthesis is increased by the vitamin¹⁷⁻²¹. Resolution of this problem requires specific antisera to an intestinal CaBP preparation from a mammalian species in which vitamin D deficiency is readily produced.

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Factors involved in initiation of haemoglobin synthesis can be phosphorylated *in vitro*

RABBIT reticulocytes, after incubation in a nutritional medium with radioactive inorganic phosphate, contain a significant amount of covalently bound phosphate. Approximately 65% of this phosphate is attached to ribosome-associated proteins which are released into the 0.5 M KCl wash fraction from ribosomes; the remainder is bound to ribosomal protein¹. The high salt wash fraction contains various proteins required for the initiation and maintenance of protein synthesis. To analyse the effects of phosphorylation on the regulation of protein synthesis, purified initiation factors from rabbit reticulocytes have been examined for phosphate-acceptor activity through the use of cyclic AMP-regulated and cyclic nucleotide-independent protein kinases. Here we describe the phosphorylation of two factors previously shown to be involved in the initiation of haemoglobin synthesis. Both IF-MP, the Met-tRNA_i binding factor, and IF-M2A, a ribosome-dependent GTPase activity, are modified by a cyclic nucleotide-independent protein kinase isolated from rabbit reticulocytes. Initiation factors IF-M1 (ref. 2), IF-M2B_a (ref. 3), IF-M2B_b (ref. 3), IF-M3 (ref. 4) and IF-M4 (ref. 5) are not phosphorylated by this enzyme.

The cyclic nucleotide-independent protein kinase activities in the post-ribosomal supernatant fraction were isolated by chromatography on DEAE-cellulose (Fig. 1). Enzyme activity was monitored by incorporation of radioactive

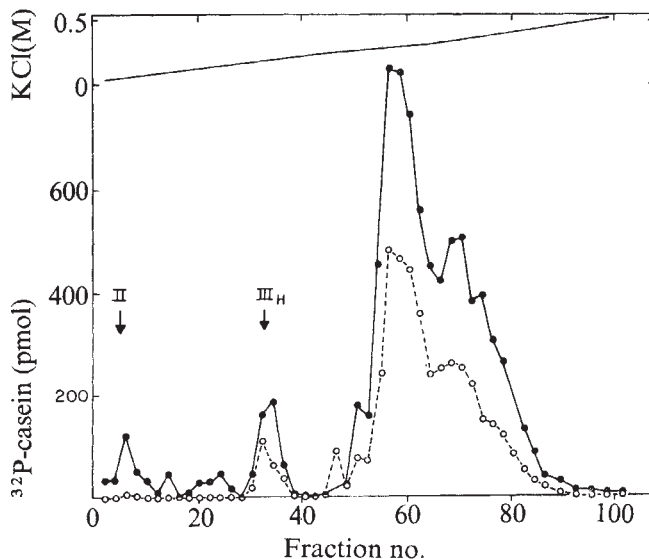


Fig. 1 Isolation of cyclic nucleotide-independent protein kinase activity from rabbit reticulocytes by chromatography on DEAE-cellulose. The post-ribosomal supernatant fraction was prepared and chromatographed on DEAE-cellulose as previously described⁶ except that 600 mg of protein was added, and elution was with a 600-ml linear KCl gradient ranging from 0 to 0.5 M KCl. Protein kinase activity was assayed using γ -³²P-ATP (●) and γ -³²P-GTP (○) as previously described⁶. The activity referred to as III_c in the text comprises fractions 54-79. Fractions 54-62, 67-72 and 74-79 were pooled individually, dialysed against 20 mM Tris-HCl, pH 7.5, 6 mM mercaptoethanol and 1 mM EDTA and stored at 4 °C.

phosphate from γ -³²P-ATP and γ -³²P-GTP into casein. The major protein kinase activity (III_c) elutes between 0.24 and 0.34 M KCl and is observed to contain several chromatographically different species. The protein kinase activity was pooled into three fractions as indicated in the legend to Fig. 1, and the enzyme activity in each of these fractions was analysed as a function of increasing concentrations of monovalent and divalent cations and for substrate specificity. No differences were observed among the three fractions (data not shown). The DEAE-cellulose column fractions were also analysed for cyclic AMP-regulated protein kinase activity using histone IIA (Sigma) as substrate in the presence of 1.4×10^{-6} M cyclic AMP. The regions where these two protein kinases eluted are indicated by arrows in Fig. 1. Further chromatography of the cyclic AMP-regulated protein kinases resolved these enzymes from cyclic nucleotide-independent activities⁶.

Highly purified initiation factors from rabbit reticulocytes were incubated with the cyclic AMP-regulated and cyclic nucleotide-independent protein kinase activities using either radioactive ATP or GTP as the phosphate donor. Phosphate-acceptor activity of the initiation factors was demonstrated by electrophoresis of the incubation mixture on polyacrylamide gels containing sodium dodecyl sulphate (SDS) followed by autoradiography. IF-MP was phosphorylated as shown in Fig. 2. This initiation factor has been

Table 1 Effect of cyclic AMP and cyclic GMP on phosphorylation of IF-MP and IF-M2A

	IF-MP		IF-M2A	
	ATP	GTP	ATP	GTP
	³² P incorporated (pmol μ g ⁻¹)			
Protein kinase (67-72)	2.7	1.9	11.6	8.0
+ cyclic AMP	2.4	1.9	13.6	9.7
+ cyclic GMP	2.5	2.1	12.1	10.7

The stained initiation factors obtained after polyacrylamide gel electrophoresis as described in Figs 2 and 3 were excised, dried and counted in a Beckman liquid scintillation counter with a toluene cocktail mixture.

purified to homogeneity⁷, and has been shown to form a ternary complex with Met-tRNA_i and GTP⁸. It is composed of three subunits which migrate with molecular weights of 57,000, 53,000 and 38,000 in polyacrylamide gels containing SDS⁹. The subunit with a molecular weight of 53,000 is phosphorylated by the cyclic nucleotide-independent protein kinase (III_C) using either ATP or GTP. With the reaction conditions used, 0.45 mol of phosphate were incorporated per mol of IF-MP, assuming a molecular weight of 180,000 (ref. 7). IF-MP is not modified by cyclic AMP-regulated protein kinases II or III_H.

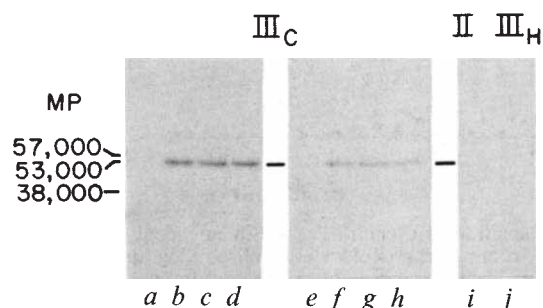


Fig. 2 Phosphorylation of IF-MP. IF-MP (1.4 μ g) was phosphorylated in 0.045 ml of reaction mixture containing 50 mM Tris-HCl, pH 7.0; 10 mM MgCl₂, 0.14 mM γ -³²P-ATP (0.2 Ci mmol⁻¹) or γ -³²P-GTP (0.12 Ci mmol⁻¹); 1.4×10^{-6} M cyclic nucleotide (where indicated), and protein kinase. The reaction contained 7 enzyme units (EU) of III_C (fractions 74–79), 25 EU of II, (further purified by chromatography on phosphocellulose⁶), or 20 EU of III_H (purified further by chromatography on carboxymethyl Sephadex). A unit of protein kinase activity has been defined previously⁶. Incubation was for 20 min at 30 °C and terminated by the addition of SDS in sample buffer. Slab gel electrophoresis was performed using the gel system of Laemmli²¹ as described by Ames²² with the following modifications: the ratio of acrylamide to *bis*-acrylamide was 22.2 : 0.6; the electrode buffer contained twice the concentration of Tris-HCl and glycine; and the resolving gel and the stacking gel acrylamide concentrations were 10% and 5% respectively. Electrophoresis was at room temperature for 0.5 h at 100 V followed by 3.5 h at 150 V. The gels were stained in Coomassie brilliant blue, destained and autoradiographed for 3–4 h against Kodak No-Screen medical X-ray film. Samples (a–d) contained ATP and III_C. a, IF-MP omitted; b, no additions; c, plus cyclic AMP; d, plus cyclic GMP. Samples (e–h) contained GTP and III_C. e, IF-MP omitted; f, no additions; g, plus cyclic AMP; h, plus cyclic GMP. i, Contained ATP, cyclic AMP, and II; j, contained ATP, cyclic AMP and III_H.

IF-M2A which catalyses a ribosome-dependent GTPase activity was also modified by the cyclic nucleotide-independent protein kinase activity that phosphorylates IF-MP (Fig. 3). This initiation factor has been observed to stimulate protein synthesis using either a natural or artificial template and has been purified to homogeneity¹⁰. As observed in the autoradiogram, either ATP or GTP can be the phosphate donor. With optimal incubation conditions, approximately two phosphoryl moieties can be incorporated per polypeptide of molecular weight 125,000, and both serine and threonine residues are modified (data not shown). IF-M2A is not modified by either of the cyclic AMP-regulated protein kinases.

The effects of cyclic AMP and cyclic GMP on the phosphorylation of both initiation factors are shown qualitatively by autoradiography in Figs 2 and 3. To quantify the effects of these nucleotides on the phosphorylation of the initiation factors, the radioactive proteins were excised from the gel, dried and counted in a liquid scintillation counter. Table 1 shows that cyclic AMP and

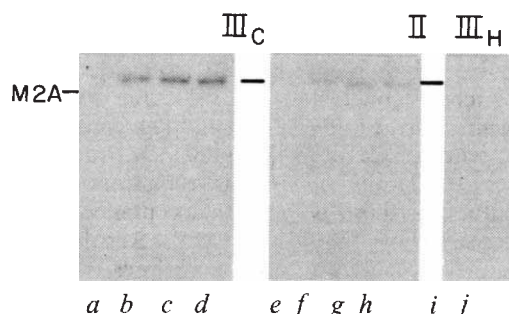


Fig. 3 Phosphorylation of IF-M2A. The procedures were described for Fig. 2 except that 1.5 μ g of homogeneous IF-M2A was reacted with 3 EU of protein kinase III_C (fractions 67–72) in a final volume of 0.025 ml for 10 min. The stacking gel contained 2.5% acrylamide and the voltage for the first 0.5 h of the run was 75 V. For identification see Fig. 2.

cyclic GMP neither stimulate nor inhibit the phosphorylation of IF-MP and IF-M2A at 10^{-6} M. In the conditions of the reaction, ATP is slightly more effective as a phosphate donor than GTP. Both the ATP and GTP were examined by thin-layer chromatography on PEI cellulose and found to be chromatographically pure with regard to contaminating radioactive nucleotides. Thus, both nucleotide triphosphates are authentic phosphate donor compounds in the reaction. Both initiation factors are modified by the three chromatographically different fractions of protein kinase which comprise III_C. These three fractions show little or no difference in the extent to which they phosphorylate the initiation factors.

Our results provide the first evidence of a cyclic nucleotide-independent phosphorylation of initiation factors which are involved in the complex process of protein synthesis. The phosphorylation of a single 40S ribosomal protein in rabbit reticulocytes has been shown to be controlled by cyclic AMP¹¹. Phosphorylation of the same protein in rat liver increases considerably when the liver is forced to regenerate¹² or in conditions of experimental diabetes¹³.

Evidence suggests that the role of haemin in the regulation of protein synthesis is mediated by phosphorylation. In the absence of added haemin, inhibition of protein synthesis in a reticulocyte lysate occurs at chain initiation with a concomitant decrease in binding of Met-tRNA_i to 40S ribosomal subunits^{14,15}. This inhibition is potentiated by the addition of ATP^{16,17}. Inhibition produced by haemin deprivation is prevented in the reticulocyte lysate by high concentrations of GTP^{16,17}, cyclic AMP^{17,18} and various purines¹⁸, and by the addition of IF-MP¹⁹. A protein kinase activity has been identified with the purified inhibitor fraction²⁰. Since the protein kinase activity described here makes use of both ATP and GTP as substrate, it is questionable whether this enzyme has the requisite properties for the translational inhibitor. But we have recently isolated another cyclic nucleotide-independent protein kinase activity which initially copurifies with cyclic AMP-regulated protein kinase III_H. This cyclic nucleotide-independent activity modifies the small subunit of IF-MP and may be the haem-controlled repressor (S.M.T., J.A.T., B.S. and W.C.M., in preparation).

Thus there are at least two possible mechanisms for the control of translation by phosphorylation—cyclic AMP-regulated modification of the 40S ribosomal subunit and cyclic nucleotide-independent phosphorylation of the initiation factors. We are analysing the differential phosphorylation of the two factors and the 40S ribosomal subunit in order to understand the molecular basis for the control of protein synthesis by phosphorylation.

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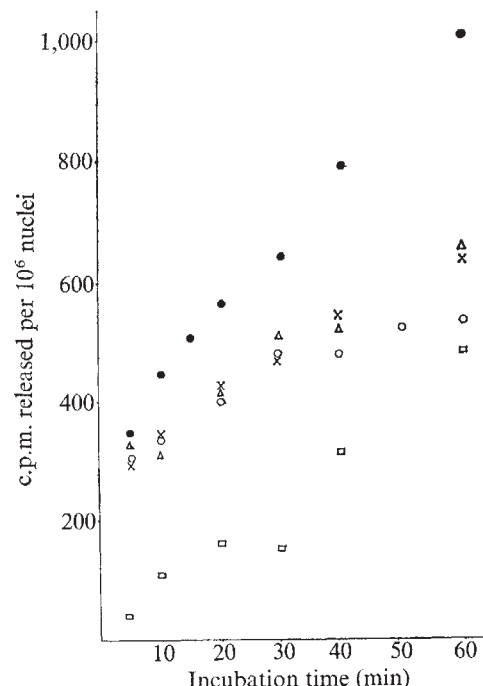


Fig. 1 Time course of RNA release from isolated nuclei. All experiments were carried out at 35 °C in the following medium¹³: 50 mM Tris-HCl, pH 7.6, 25 mM KCl, 2.5 mM MgCl₂, 0.3 mM MnCl₂, 0.5 mM CaCl₂, 5.0 mM NaCl, 2.5 mM K₂HPO₄, 5.0 mM spermidine and 2.0 mM dithioerythritol. ○, Control; ●, release on addition of 2.0 mM ATP; ×, release on addition of 2.0 mM EDTA; △, release on addition of 2.0 mM adenylyl-β,γ-methylene diphosphonate (AMP-PCP); □, difference between ATP-stimulated and control values. After 30 min incubation, 74±4% of the counts released in the control and 90±5% of the additional counts released in the presence of ATP were precipitable with 5% TCA. There was no significant difference between the release in the presence of EDTA and that in the presence of AMP-PCP; indicating that, contrary to the finding of Raskas⁷, the effect of AMP-PCP is solely attributable to its chelating action. The effect of ATP, however, is clearly not wholly attributable to chelation.

Evidence for involvement of nuclear envelope nucleoside triphosphatase in nucleocytoplasmic translocation of ribonucleoprotein

A NUCLEOSIDE triphosphatase activity showing broad substrate specificity has been found in nuclear envelope isolated from rat and pig liver, *Tetrahymena* macronucleus, and cultured SV-3T3 fibroblasts using the method of Harris and Milne¹, and has been extensively characterised in this laboratory. There is evidence that in purified nuclear envelopes there is only one enzyme which catalyses nucleoside triphosphate hydrolysis: the activities observed with different substrates and metal ions are not additive, and they show a single pH optimum, the value of which varies with the source of the envelopes². This enzyme seems to be localised in the nuclear pore complexes³. Several reports⁴⁻¹³ have indicated that ATP markedly stimulates the release of mRNA from isolated nuclei, but no evidence has been presented to suggest that this stimulation necessarily requires hydrolysis of the ATP. In this paper, however, we present evidence that the nucleoside triphosphatase is an essential component of the mechanism of nucleocytoplasmic translocation of ribonucleoprotein.

SV-3T3 cells were grown in Dulbecco's modified Eagle's medium (Flow Laboratories) and labelled for 6 h with 10 μCi ¹⁴C-orotic acid (61 mCi mmol⁻¹, Amersham). The cells were homogenised in 10 mM Tris-HCl, pH 7.4, containing 1 mM MgCl₂, and centrifuged through 2.2 M sucrose in the same Tris-MgCl₂ buffer at 100,000g for 1 h at 0 °C. The pellet of nuclei was substantially free of 5' nucleotidase

(EC 3.1.3.5), Na/K-stimulated Mg-ATPase (EC 3.6.1.4) and succinate dehydrogenase (EC 1.3.99.1), and no organelle contamination was visible under phase contrast (B.McC., P.S.A. and J. F. Lamb, unpublished). Digestion of the nuclei in 0.1 M NaOH and counting in a Packard Tri-Carb scintillation spectrophotometer using NE 250 scintillant (NEM) showed ¹⁴C incorporation of 5,000±1,000 c.p.m. per 10⁶ nuclei. In the presence of 50 μg ml⁻¹ proflavine, orotic

Table 1 Specificity of the nucleoside triphosphatase and of nucleoside-triphosphate-stimulated RNA release from isolated nuclei for various divalent cation-nucleoside triphosphate complexes

Substrate	a	b
Mg.ATP ²⁻	16.7±1.1	438±21
Mg.GTP ²⁻	18.0±1.1	450±22
Mg.UTP ²⁻	7.9±0.5	219±15
Mg.CTP ²⁻	7.2±0.5	153±13
Ca.ATP ²⁻	13.4±1.1	350±19
Mn.ATP ²⁻	11.5±1.1	306±18
Zn.ATP ²⁻	1.3±0.4	22±10
Be.ATP ²⁻	Zero	Trace

a, Nucleoside triphosphatase activity (μmol inorganic phosphate released per h per mg protein). b, Stimulated RNA release (c.p.m. per h per 10⁶ nuclei)—that is, the difference between counts released in the presence and in the absence of the nucleoside triphosphate. For the release experiments, nuclei were incubated at 35 °C in 0.88 M sucrose containing either 5 mM MgCl₂, CaCl₂, MnCl₂, ZnCl₂ or BeCl₂, and the 5% TCA precipitate of the supernatant was counted as described in the text⁴. The results in column b are in good agreement with those of Ishikawa *et al.*⁴.

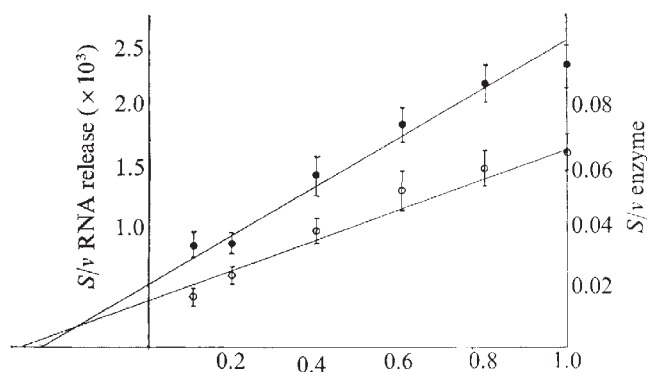


Fig. 2 Eadie plots comparing dependence of rates of nucleoside triphosphatase reaction (○) and ATP-stimulated RNA release from nuclei (●) on ATP concentration. For the left-hand ordinate (S/v RNA release), the v term has been taken as the difference in label released (c.p.m. per h per 10^6 nuclei) in the presence and absence of ATP at each concentration. For the right-hand ordinate (S/v enzyme), the v term is the nucleoside triphosphatase activity (μmol phosphate released per h per mg envelope protein). Enzyme assays were carried out as described in the text, the free Mg^{2+} concentration being maintained at 0.2 mM throughout^{2,18}. RNA release experiments were carried out essentially as described in Fig. 1; but to avoid spurious results due to the chelating action of ATP, EDTA was added to make the total ATP+EDTA concentration 1.0 mM throughout. K_m values obtained are 0.26 ± 0.05 mM for the nucleoside triphosphatase, and 0.30 ± 0.09 mM for the ATP-stimulated RNA release. The agreement between the behaviour of the two processes is again close.

acid incorporation was reduced to 850 c.p.m. per 10^6 nuclei, indicating that most of the label was taken up into the nuclear RNA¹⁴.

The media described by Ishikawa *et al.*⁴ and by McNamara *et al.*¹³, which maintain the nuclei in a stable and essentially disaggregated state, were used in the RNA release experiments; the yeast RNA, ATP-generating system and cytoplasmic protein¹³ were omitted. Volumes (0.75 ml) of a suspension containing approximately 5×10^6 nuclei ml^{-1} , with and without ATP (routinely 2.0 mM) and other reagents, were incubated at 35 °C for various times. The suspension was centrifuged at 3,000g for 5 min to remove nuclei, and 0.4-ml aliquots of the supernatant counted as before.

The nucleoside triphosphatase activity proved resistant to all attempts at solubilisation. Enzyme assays were therefore carried out by incubating whole nuclear envelopes in 25 mM triethanolamine-HCl buffer, pH 7.7, with ATP and MgCl_2

at 35 °C for 30 min, and determining inorganic phosphate release¹⁵. In experiments in which the effects of inhibitors were investigated, the nuclei or envelopes were preincubated with the inhibitor for 5 min, and solvent blanks were used as controls.

Substrate specificity, kinetic behaviour and inhibition properties of the ATP-stimulated RNA release were investigated and compared with the corresponding properties of the nucleoside triphosphatase, to establish whether there was a causal connection between the two processes. The effect of ATP on RNA release from the nuclei is shown in Fig. 1: 25–30% of the label released in the absence of exogenous ATP is attributable to low molecular weight (TCA-soluble) material; the remainder presumably represents leakage of RNA from the nuclei, although the possibility that traces of endogenous ATP may contribute to the background cannot be ruled out. Preliminary isotachophoretic measurements indicate not more than 20 nmol ATP per 10^6 freshly prepared nuclei.

Table 1 shows that replacement of other nucleoside triphosphates for ATP and of other divalent cations for Mg^{2+} still resulted in stimulation of RNA release from the nuclei. There is a striking correlation between this stimulated release and the rate of hydrolysis catalysed by the nucleoside triphosphatase for all divalent cation-nucleoside triphosphate complexes investigated. The nuclear envelope contains an active glucose-6-phosphatase (EC 3.1.3.9) (ref. 3), but replacement of ATP by 2.0 mM glucose-6-phosphate was not attended by significant stimulation of the RNA release.

Table 2 compares the effects of inhibitors on the nucleoside triphosphatase and on the ATP-stimulated release of RNA. Again, the correlation is striking, particularly so because the inhibitor concentrations for half-maximal inhibition were similar for both processes. The dependence of the rate of ATP-stimulated RNA release on ATP concentration approximated to Michaelis-Menten kinetics, with a K_m' value (2.8×10^{-4} M) close to that for the nucleoside triphosphatase (Fig. 2). For all properties investigated, therefore, there was a close correspondence between the behaviour of the two processes, indicating a likely causal connection.

There is strong evidence that the RNA released as a ribonucleoprotein complex into the supernatant in the conditions described in this paper is predominantly mRNA. Although the possibility of some contamination with heterogeneous nuclear RNA cannot be ruled out, normal nuclear restriction of RNA has been clearly demonstrated using the medium described by McNamara *et al.*¹⁶ and Ishikawa *et al.* note that the RNA released in the conditions they use in the presence of ATP is largely incorporated into polyosomes¹⁷. If these *in vitro* conditions, which have been used in the present investigation, can be accepted as a reasonable facsimile of conditions *in vivo*, then the results we have described here strongly suggest that the nuclear envelope nucleoside triphosphatase constitutes an essential part of the mechanism of nucleocytoplasmic ribonucleoprotein translocation. Detailed kinetic studies of the enzyme have been carried out in this laboratory¹⁸, with a view to illuminating the molecular mechanism of this translocation process.

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Table 2 Effects of various inhibitors on the nuclear envelope nucleoside triphosphatase and on ATP-stimulated RNA release from isolated nuclei

Inhibitor	a	b
Quabain 2.0 mM	100 $\pm$ 8	100 $\pm$ 9
Gramicidin 50 $\mu\text{g ml}^{-1}$	100 $\pm$ 6	100 $\pm$ 3
Cycloheximide 50 $\mu\text{g ml}^{-1}$	100 $\pm$ 4	100 $\pm$ 7
Oligomycin 5 $\mu\text{g ml}^{-1}$	85 $\pm$ 5	82 $\pm$ 8
50 $\mu\text{g ml}^{-1}$	60 $\pm$ 10	52 $\pm$ 6
Quercetin 5 $\mu\text{g ml}^{-1}$	50 $\pm$ 6	35 $\pm$ 5
15 $\mu\text{g ml}^{-1}$	Trace	Trace
AMP.PCP 2.0 mM	100 $\pm$ 7	100 $\pm$ 6
ATP γ S 1.0 mM	50 $\pm$ 10	38 $\pm$ 6
2.0 mM	16 $\pm$ 8	Trace

a, Nucleoside triphosphatase activity; b, rate of RNA release. All values are expressed as percentage of control ± 1 s.e.m. ATP γ S, Adenosine-5'-O-(3)-thiotriphosphate. ATP analogues were obtained from Boehringer; other inhibitors from Sigma.

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Unexpected occurrence of an aminoacylated nucleoside in mammalian tRNA^{Tyr}

MANY tRNAs contain a hypermodified nucleoside at the 3' side of their anticodons¹. The modifications at this position are essential for tRNA function in ribosome binding and amino acid transfer^{2–4}, and cause inability to base pair according to the Watson–Crick scheme⁵. A striking correlation between the codon response of the tRNA and the nature of this hypermodified nucleoside has been observed: all known sequences of tRNAs (*Escherichia coli*), with codons beginning with uracil, contain N⁶-(Δ²-isopentenyl)-2-methylthio-adenosine at the 3' side of the anticodon^{6–8}, whereas tRNAs with codons starting with adenine, contain an aminoacylated nucleoside, such as N-[N-(9-β-D-ribofuranosyl-purin-6-yl)carbamoyl] threonine (t⁶A) or the methyl derivative (mt⁶A) at this position^{9,10}.

The same distribution of these isopentenylated and aminoacylated nucleosides has also been observed in yeast^{11–13} and mammals^{14,15}. This striking regularity led to the widely accepted postulate that N⁶-(Δ²-isopentenyl-adenosine) (i⁶A) or the methylthiolated derivative might have a specific role in the recognition of codons starting with uridine¹, whereas t⁶A is thought to prevent pairing between uridine in the third position of the anticodon and guanine in the first position of the codon¹⁶.

The Tyr-tRNAs read the UAX-codons and contain in *E. coli*^{17,18}, *Torulopsis utilis*¹⁹, and yeast¹¹ i⁶A next to the anticodon, as expected. Tyr-tRNA from rat liver, however, chromatographs very differently from the i⁶A-containing species on partition chromatography columns²⁰, and elutes at about the same position as tRNA_{3^{ser}} which contains the hydrophilic nucleoside mt⁶A (ref. 15). This chromatographic behaviour

of tRNA^{Tyr} (rat liver) indicates the presence of a polar nucleoside in the anticodon loop of this molecule. As mentioned, t⁶A was thought to be present exclusively in tRNAs coding for AXX codons, so we did not expect it in tRNA^{Tyr}, which codes for UAX. Here we report the unexpected occurrence of t⁶A in tRNA^{Tyr} from rat liver.

tRNA^{Tyr} was purified from crude rat liver tRNA (ref. 21) using two different fractionation procedures. The first involved fractionation on BD-cellulose column chromatography²² at pH 4.5. The tRNA^{Tyr}-enriched fractions were then separated on a DEAE-Sephadex A 50 column²³ at pH 7.3, further purified on a BD-cellulose column in 7 M urea²⁴ at pH 4.5, and finally separated on a reversed phase column (system 5)²⁵. The second purification method consisted of fractionation on a BD-cellulose column in 7 M urea at pH 4.5, then the tRNA^{Tyr}-enriched fractions were phenoxylated²⁶ and rechromatographed using the same chromatography system. The latter method takes advantage of the fact that a modified uridine in tRNA^{Tyr} reacts specifically with the phenoxylating reagent leading to a more lipophilic elution position of the derivatised tRNA, so that tRNA^{Tyr} can be separated rapidly from non-derivatised material²⁷. From the elution profile of other tRNAs one can conclude that the structures of hypermodified nucleosides in the anticodon loop are not affected by this method.

After enzymatic hydrolysis to nucleosides, pure fractions of tRNA^{Tyr} (rat liver) were analysed by two-dimensional thin-layer chromatography on cellulose-coated aluminium foils, as described previously²⁸. The nucleosides were identified by their characteristic chromatographic behaviour and by their ultraviolet absorption spectra at pH 1, 6 and 12, as well as by their characteristic fluorescence and phosphorescence at 77 K (ref. 29). The two-dimensional chromatogram of a hydrolysate of rat liver tRNA^{Tyr} is shown in Fig. 1. With the exception of the chemically modified uridine derivative in case of the phenoxylated tRNA, identical patterns were obtained by analysis of fractions from the two different purification methods. The following nucleosides were found: adenosine, 13–15; cytidine, 19–20; guanosine, 20–22; uridine, 10–11; pseudouridine, 3–4; ribothymidine, 1; dihydrouridine, 3–4; 1-methyladenosine, 1; 5-methylcytidine, 1; 1-methylguanosine, 1; N²-methylguanosine, 1; N²,N²-dimethylguanosine, 1; 7-methylguanosine, 1; and an unknown uridine derivative (U*) which reacts with the phenoxylating reagent. Neither i⁶A, nor one of its derivatives, was found in the hydrolysate. Instead, one residue of the nucleoside t⁶A, which is found exclusively at the 3' position adjacent to the anticodon, was detected. Since t⁶A was thought to be present only in tRNAs coding for AXX codons and therefore should not occur in the tyrosine-specific tRNA, we proved the identity of t⁶A by the following extensive criteria. (1) The ultraviolet absorption spectra of the compound at different pH showed the same characteristics as those of the synthetic t⁶A (Fig. 2). (2) After alkaline hydrolysis of t⁶A from tRNA^{Tyr} with KOH (0.3 M, 16 h, 37 °C)

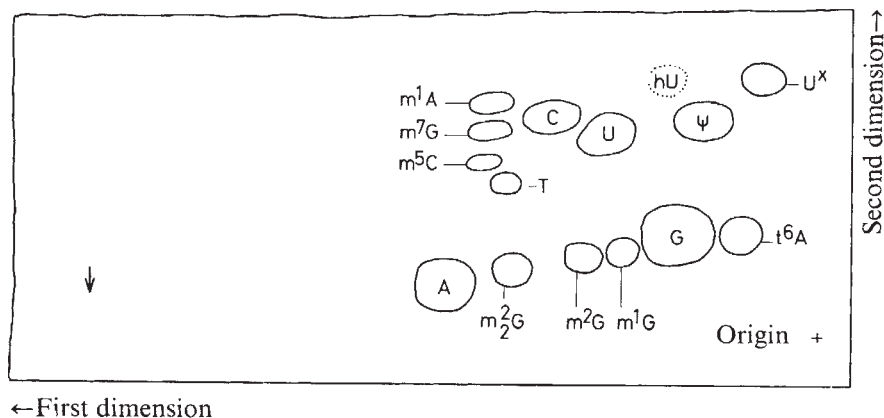


Fig. 1 A two-dimensional thin-layer chromatogram of the digest of tRNA^{Tyr}. 2–3 A₂₆₀ units of the desalted tRNA^{Tyr} were degraded enzymatically overnight at 37 °C by 25 μg pancreatic RNase (Boehringer GmbH, Mannheim), 30 μg snake venom phosphodiesterase (Worthington) and 25 μg alkaline phosphatase (Worthington, Code BAPF) in a siliconised test tube containing 0.02 M ammonium formate (pH 7.6) and 5 × 10⁻⁴ M MgCl₂ in 0.2 ml (ref. 28). The digest was subsequently treated for 2 h at 37 °C with 5 μg ribonuclease T₁ (Calbiochem) and then applied on a cellulose-coated aluminium foil, as described previously²⁸. The solvent systems used (see Table 1) were solvent C in the first dimension and solvent D in the second. The arrow indicates the established position of isopentenyladenosine which is absent in this hydrolysate.

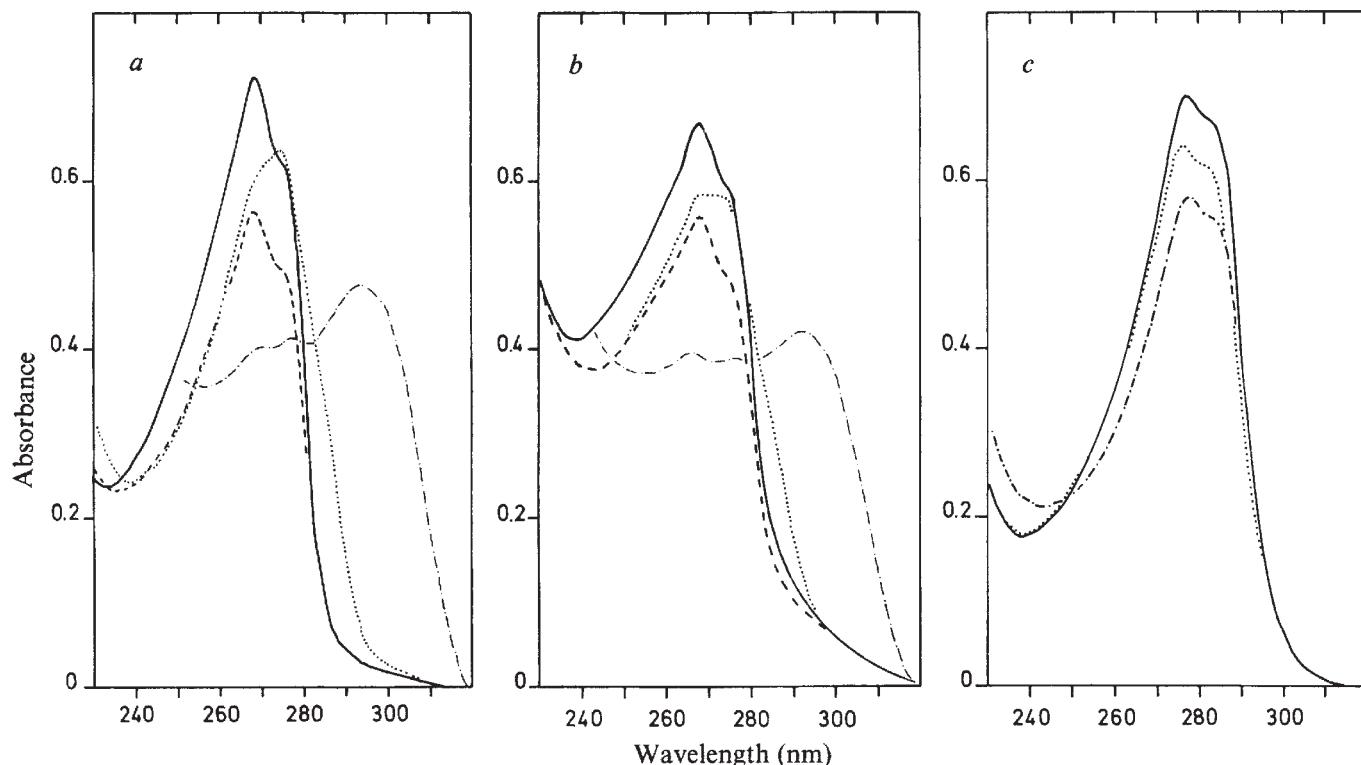


Fig. 2 Ultraviolet absorption spectra of aminoacylated nucleosides. *a*, Synthetic t^6A ; *b*, t^6A from rat liver tyrosine tRNA; *c*, synthetic mt^6A pH 1; — pH 6; --- pH 12; -.- pH 13.

and removal of the potassium with perchloric acid, the ultraviolet absorption spectra of the hydrolysate was identical to that of adenosine. In addition, the degradation product also showed mobility identical to adenosine on three different thin-layer chromatographic systems. (3) Automatic amino acid analysis after degradation of the compound in $Ba(OH)_2$ (0.1 M, 20 min in a boiling water bath and 16 h at 37 °C, followed by removal of Ba^{2+} with sulphuric acid) yielded equal amounts of threonine and serine. Control experiments using synthetic t^6A established that in these hydrolysis, considerable amounts of threonine were degraded to serine. (4) The chromatographic properties of t^6A from tRNA^{Tyr} in four different solvent systems were identical to those of synthetic t^6A and different from either its glycine analogue g^6A or mt^6A (Table 1). (5) Chromatography on DEAE-cellulose at pH 8–8.8 showed that the compound had a negative charge like t^6A and differed therefore from another aminoacylated derivative which had been isolated from tRNA₂^{Met} (*E. coli*)¹ and which would be uncharged at this pH.

These findings clearly prove the presence of the aminoacylated nucleoside t^6A in tRNA^{Tyr} (rat liver). Analysis of

tRNA^{Tyr} from the silk worm (*Bombyx mori*), showed that in tRNA^{Tyr} from this organism, i^6A is also replaced by t^6A , indicating that t^6A in tRNA^{Tyr} seems to be a common feature of higher organisms—thus contrasting strongly the situation in *E. coli*^{6–8} and yeast^{11–12}.

It is particularly interesting that the nature of the base adjacent to the anticodon of tRNAs coding for UXX codons is very different in *E. coli* and in mammals. In *E. coli* this position is always occupied by $N^6-(\Delta^2\text{-isopentenyl})\text{-2-methylthio-adenosine}$, whereas in higher organisms there were detected (in addition to isopentenyl-adenosine) wybutosine (Y) in tRNA^{Phe} (yeast)³⁰, 1-methyl-guanosine in tRNA₃^{Leu} (yeast)³¹, adenosine in tRNA^{Trp} (yeast)³² and derivatives of wybutosine in tRNA^{Phe} of wheat germ³³ and mammals^{34–37}. This may indicate that the problem of recognition of these tRNAs by the ribosome might be solved differently in prokaryotes and higher organisms.

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Table 1 Chromatographic mobilities of aminoacylated nucleosides compared with t^6A from tRNA^{Tyr} (rat liver) in four different solvent systems

Nucleoside	R_F values ($\times 100$) in solvent			
	A	B	C	D
t^6A from tRNA ^{Tyr} (rat liver)	49	53	10	30
t^6A (synthetic)	49	53	10	30
mt^6A	58	59	13	27
g^6A	38	48	5	10
adenosine	54	74	42	11

The following solvent systems (cited in ref. 28) were used: A, *n*-propanol-concentrated ammonium hydroxide–water (90:45:15, by volume); B, isobutyric acid–ammonium hydroxide (0.5 M)–EDTA (0.1 M) (100:60:1.6, by volume); C, 1-butanol–isobutyric acid–water–ammonium hydroxide (25%) (150:75:50:5, by volume); D, saturated ammonium sulphate–sodium acetate (0.1 M) (pH 6)–isopropanol (79:19:2, by volume).

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Primary processes in photochemistry of rhodopsin at room temperature

Most of the information concerning the primary photochemical events in vision has been derived from low temperature work¹⁻⁵. Recently, pulsed laser excitation methods with nanosecond⁶⁻⁸ or picosecond⁹ time resolution have been applied, in an attempt at clarifying the primary act at physiological temperatures. These studies showed that prelumi-rhodopsin PLR; or bathorhodopsin, which is the primary photoproduct at 77 K, is also formed at room temperature, with a rise time of less than 6×10^{-12} s (ref. 9) and a half life of $\sim 10^{-7}$ s (refs 6-9). The problem remained, however, as to whether other primary transients, in addition to PLR, are formed at room temperature⁸. It is also important to establish if PLR is the unique precursor of the visual sequence at physiological temperatures, ruling out direct routes to subsequent intermediates—for example, lumirhodopsin (LR)—circumventing PLR. The present pulsed Nd laser study has attempted to clarify the above points, essentially testing the applicability of the low temperature photochemical picture (refs 10 and 11, and T.R., B. Honig, M.O. and T. Ebrey, unpublished) in physiological conditions.

The Nd laser photolysis system has been described previously¹². Photolysis of the rhodopsin samples by the monitoring light was minimised by using short (10^{-2} s) exposures (obtained with a synchronised camera shutter) and appropriate combinations of Corning glass filters. Nanosecond measurements (with the monitoring source used in an intense pulsed mode) were not carried out in the 480-520-nm range, where no such filter combinations could be found, to reduce bleaching by the monitoring light (to less than $\sim 5\%$) without lowering its intensity (I) below the minimum required¹³. The range between 520 and 550 nm was also avoided because of interference from light scattered by the laser. The above limitations on I impose serious restrictions on the signal-to-noise ratio (S/N) of the present nanosecond laser experiments (Fig. 1a and b, S/N = 5-10). A more intense monitoring pulse, however, significantly improving the accuracy of the measurements, pumps the system to an undefined mixture of rhodopsin and its bleaching products, preventing a quantitative analysis of the net effects due

to laser excitation of rhodopsin. Highly pure rhodopsin solutions in 0.067 M phosphate buffer (2% Ammonyx LO) were prepared as described previously¹⁴⁻¹⁶.

Characteristic oscillograms, showing the main transient decay patterns after pulsed (530 nm, 40 ns) Nd laser excitation of rhodopsin solutions at 22 °C, are shown in Fig. 1. Oscillograms (a-d and f) were recorded using a pulse energy of ~ 20 mJ which is sufficiently high to reach saturation (absorbance changes independent of the pulse intensity), reflecting the attainment of a photostationary equilibrium between the primarily excited rhodopsin and photoproducts formed during the pulse (for example, PLR)¹². Oscillogram e was recorded with a reduced pulse intensity, as indicated by the drop in the transient absorbance relative to, for example, f. Figure 1a and b shows that the initial changes at the end of the pulse are followed by decay and growing-in processes, exhibiting a half life of ~ 100 ns. These fast events are followed by much slower changes in the μ s range (Fig. 1c and d). The corresponding difference spectra, recorded 50 ns, 700 ns and 1 ms from the origin of the pulse (Fig. 2), are proportional to the differences $D_T^\lambda - D_R^\lambda$, where D_R^λ is the drop in the absorbance of rhodopsin due to photobleaching and D_T^λ is the (time-dependent) absorbance of photoproducts present after the pulse. The dotted curves in the range where experimental points are not available were estimated as described below.

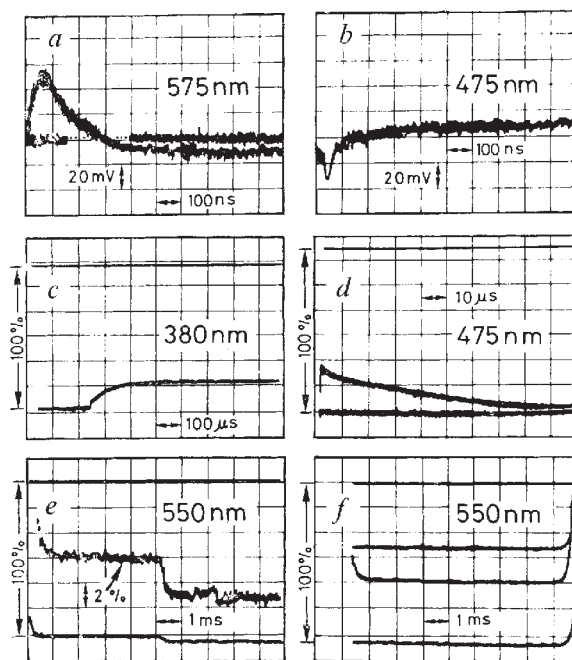


Fig. 1 Characteristic oscillograms in the Nd laser photolysis of rhodopsin at room temperature. The initial absorbance of the solutions at 495 nm ranged from 0.5 to 1.0. Experiments a, b, c and f were carried out using a 20-mJ pulse, whereas e was carried out using a reduced intensity (oscillator only, without amplification). Load resistors used: 50 Ω (a, b and d); 10 K Ω (c) and 100 K Ω (e and f). 100% (c-f) and 2% (f) denote the percentage of transmitted monitoring light. Upper traces in c-f were recorded in the absence of the monitoring beam. Vertical sensitivity 20 mV (a, b, d and e, central trace); 10 mV (c); 200 mV (e, bottom and upper traces); 100 mV (f). The initial light to dark deflections (not shown) in a and b were 180 mV and 170 mV, respectively. The break in the lower traces in c and e is due to firing of the laser pulse. In all other cases the laser was fired almost simultaneously with the oscilloscope trigger. In oscillogram f the lower trace denotes the transmittance of a bleached rhodopsin solution. The two central traces were recorded in an unbleached solution, without (upper) and with (lower) the laser being fired. From this information it is possible to evaluate the absorbance of the solution before pulsing and the change induced by the laser pulse between 1 and 7 ms. This enables evaluation of percentage rhodopsin bleached, as described in the text.

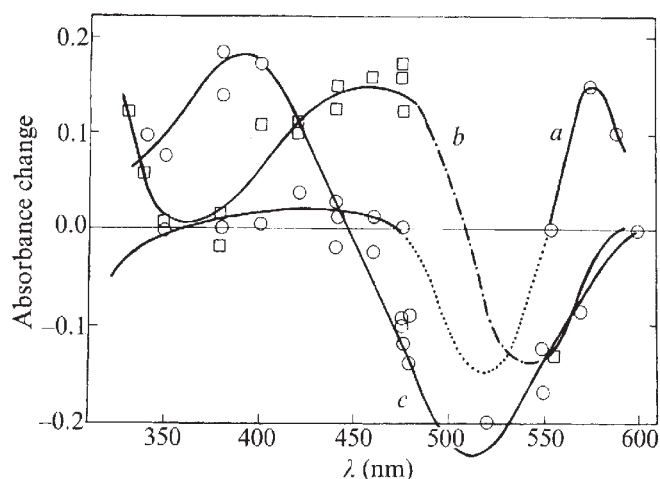
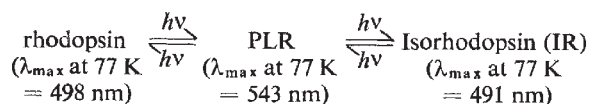


Fig. 2 Transient absorbance changes after pulsed Nd laser excitation of rhodopsin at room temperature. Data were recorded 50 ns (a), 700 ns (b) and 1 ms (c) after firing the laser. In a and b the spectral resolution is of the order of 5 nm (above 350 nm) or 10 nm (below 350 nm).

Oscillograms c and d in Fig. 1 and curve c in Fig. 2 show that after ~ 0.3 ms, bleaching in the range of the 500-nm rhodopsin band is associated with an increase in absorbance at about 380 nm, characteristic of the spectrum of meta-rhodopsin II (MII)^{3,17,18}. Similarly, the difference spectra a and b may be associated with the generation of PLR (λ_{max} at 77 K = 543 nm) and LR (λ_{max} = 490 nm). (In the present experiments we have not attempted to identify MI, the precursor of MII, the absorption spectrum of which is similar to that of LR.) A closer examination of Fig. 2 indicates, however, that the observed difference spectra cannot be rationalised only in terms of PLR, LR and MII. In all three cases the absorbance change in the 450–550-nm range is higher than that expected from the corresponding low temperature differences in extinction coefficients^{1,17,18}: $\epsilon_{\text{MII}}^{\lambda} - \epsilon_{\text{LR}}^{\lambda}$, $\epsilon_{\text{R}}^{\lambda} - \epsilon_{\text{R}}^{\lambda}$ and $\epsilon_{\text{PLR}}^{\lambda} - \epsilon_{\text{R}}^{\lambda}$, indicating that apart from PLR an additional product (X) is present at the end of the pulse which is stable at least up to the stage of MII. A new band in the neighbourhood of 450 nm was also reported by Bensasson *et al.*⁸ along with a peak at about 330 nm, not observed in the present work.

Information relevant to the identification of X was obtained by measuring its contribution, reflected by the absorbance drop at 480 nm (at the stage of MII) relative to that at 550 nm, as a function of the laser pulse intensity. Excitation below saturation (for example, Fig. 1e), when the amount of light absorption by PLR relative to rhodopsin is considerably reduced, leads to an increase in the above ratio ($D_{480}/D_{550} = 2.2 \pm 0.4$), compared with that (0.9 ± 0.3) obtained using saturating excitation energies (for example, Fig. 2c). It seems therefore that X is a secondary product, formed during the pulse because of light absorption by PLR. Thus, in view of the photoequilibrium



which is known to be established at 77 K (refs 3 and 19), it is strongly suggested that X should be identified with the 9-*cis* isomer, IR (λ_{max} at 25 °C = 485 nm)¹¹.

This suggestion may be more quantitatively tested by considering the change in absorbance after 1 ms at 550 nm (where $\epsilon_{\text{MII}} \sim 0$)

$$\Delta D_{550} = C_{\text{IR}}\epsilon_{\text{IR}}^{550} - C_{\text{MII}}\epsilon_{\text{R}}^{550} - C_{\text{IR}}\epsilon_{\text{R}}^{550}$$

Taking¹¹ $\epsilon_{\text{IR}}^{550}/\epsilon_{\text{R}}^{550} \sim 0.5$ and neglecting the contributions of

IR and rhodopsin at 380 nm (that is, approximating: $\Delta D_{380} \sim \epsilon_{\text{MII}}^{380}C_{\text{MII}}$), we have

$$\Delta D_{550} = -\epsilon_{\text{R}}^{550}(C_{\text{IR}}/2 + \Delta D_{380}/\epsilon_{\text{MII}}^{380})$$

From the data of Fig. 2 (with $\epsilon_{\text{MII}}^{380} = 4.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{R}}^{550} = 1.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) we obtain $C_{\text{MII}} = 4.5 \times 10^{-6} \text{ M}$ and $C_{\text{IR}} = 6.6 \times 10^{-6} \text{ M}$. The concentration of bleached rhodopsin is $C_{\text{IR}} + C_{\text{MII}}$ and its contribution to the difference spectra in Fig. 2, $D_{\text{R}}^{\lambda} = \epsilon_{\text{R}}^{\lambda}(C_{\text{IR}} + C_{\text{MII}})$ is shown in Fig. 3a. Addition of D_{R}^{λ} to each of the difference spectra in Fig. 2 yields curves d, e and f in Fig. 3, representing correspondingly D_{T}^{λ} (50 ns), D_{T}^{λ} (700 ns) and D_{T}^{λ} (1 ms). It can be seen that curve f can be represented by the superimposition of the known spectra^{11,17,18} of MII (Fig. 3c) and IR (Fig. 3b), in keeping with the identification of X as IR. A similar procedure shows that curves e and d are obtainable by superimposing the contribution of IR and correspondingly (allowing for a 10–20-nm temperature shift and a $\sim 10\%$ change in extinction coefficients) those of LR and PLR in amounts equal to that of MII.

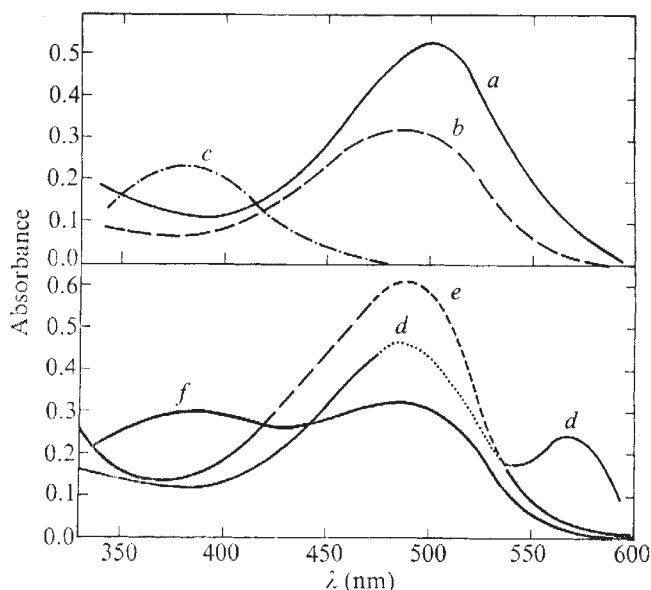


Fig. 3 a, Contribution of bleached rhodopsin (D_{R}^{λ}) to the difference spectra of Fig. 2; b and c, spectra of MII and of transient X (IR), respectively. d–f, Spectra of transients present after 50 ns (d), 700 ns (e), and 1 ms (f) evaluated from the corresponding traces in Fig. 2, as described in the text.

The above observations are consistent with PLR being the unique primary product in the photochemistry of rhodopsin at room temperature. It is also evident that it constitutes the precursor of the subsequent intermediates in the cycle (LR, MI, MII, and so on). Thus, the classical sequence of primary events as derived for rhodopsin on the basis of low temperature work^{1–5} seems to be applicable at physiological temperatures, in agreement with a conformational (for example, *cis-trans*) change as the only primary step of the visual cycle (refs 10 and 11, and T.R. *et al.*, unpublished).

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Structure of thymidyl-3',5'-deoxyadenosine

THE triethylammonium salt of the dinucleoside phosphate, thymidyl-3',5'-deoxyadenosine (TpdA) forms helical structures when aqueous solutions are allowed to evaporate¹. There are some similarities between the X-ray diffraction patterns from TpdA and those from poly(dA-dT)–poly(dA-dT), and we originally thought that the structures might be similar¹. But when the structure for poly(dA-dT)–poly(dA-dT) was proposed² it was apparent that the TpdA structure was not of the same kind. This, together with the fact that we had obtained more detailed X-ray diffraction patterns from better oriented TpdA specimens, led us to consider the different molecular model for TpdA which we describe here.

Figure 1a shows the diffraction pattern from a specimen of TpdA at ~60% relative humidity. The layer-line spacing corresponds to a periodicity of 26.4 Å and the equatorial Bragg reflections fit a hexagonal lattice with $a = 21.3$ Å. Discrete reflections are confined to the inner region of the equator—the remainder of the pattern shows continuous intensity along the layer lines. The strongest intensity is in the meridional region of the 7th layer line. The intensity on the 8th layer line is also very strong as well as the first maximum on the 1st layer line. The near meridional diffraction on layer lines 5–8 is similar to the diffraction in the same region in patterns from DNA, double-helical RNA and synthetic polynucleotides. It is mainly due to the bases and gives information about their tilt relative to the helix axis.

In the early photographs it was difficult to decide on which of the layer lines 5–8 the intensity was truly meridional and thus to decide the number of residues per turn of the helix. Better oriented specimens, tilted at 12° from perpendicular to the X-ray beam show that the intensity on the 5th and 8th layer lines is not meridional (Fig. 1b). But it is not possible to decide whether the truly meridional intensity occurs on the 6th or the 7th layer lines. Tilting a specimen at 24° from perpendicular to the X-ray beam to examine the meridional region of the 12th and 14th layer lines, it is again difficult to decide which of these two has truly meridional intensity (Fig. 1c). In fact the intensity on the 13th layer line seems to be more meridional, although this appearance could be a result of the intensity distribution on these layer lines, which is very dependent on the tilt of the bases.

Meridional intensity on the 7th and 14th layer lines would imply seven residues per turn of the helix, with a rise per residue of 3.77 Å and a rotation per residue of 51.4°. Meridional intensity on the 6th and 12th layer lines would imply six residues per turn, with corresponding translational and rotational values of 4.4 Å and 60°.

Our original idea that the TpdA structure might be similar to that of poly(dA-dT)–poly(dA-dT) would mean that the repeating unit of the structure was a pair of TpdA molecules related to each other by a twofold rotation axis perpendicular to the helix axis. The large tilt of the bases required to give the appropriate rise per residue in such a model is not however, consistent with the near-meridional intensity distribution in the diffraction pattern. We therefore considered alternative models, in which the repeating unit is one TpdA molecule and with a smaller tilt of the bases, as suggested by the near-meridional diffraction intensity. We assumed that within the dinucleoside phosphate, the two nucleosides are related by a right-handed screw, as in all other helical polynucleotide structures. We find that it is possible to construct a stereochemically acceptable model with seven TpdA molecules per turn of the helix, and which also gives a calculated Fourier transform which is in reasonably good agreement with the observed diffraction pattern (Fig. 2). A similar sixfold model does not give good agreement with observation.

The thymine of one TpdA molecule is hydrogen bonded to, and coplanar with, the adenine base of the next. This is only possible if one TpdA molecule is related to the next by a left-handed screw. The model is thus a left-handed helix, although within the dinucleoside phosphate unit, the nucleosides are related as in a right-handed helix. To use protein structure terminology, the secondary structure is right handed but the quaternary structure is left handed. Adenine and thymine are

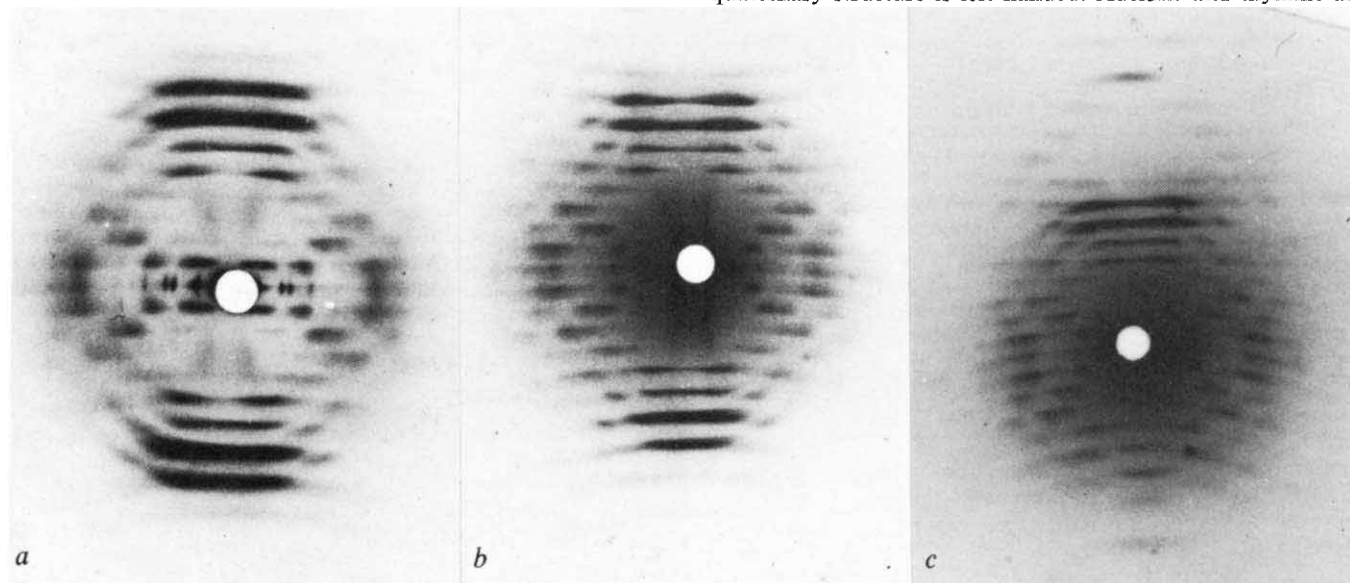


Fig. 1 X-ray diffraction patterns from TpdA specimens at ~60% relative humidity. Diffraction photographs were taken on a North American Phillips microcamera, using a Hilger and Watts semimicrofocus X-ray generator. a, Specimen approximately perpendicular to X-ray beam; b, specimen tilted through 12°; c, specimen tilted through 24°.

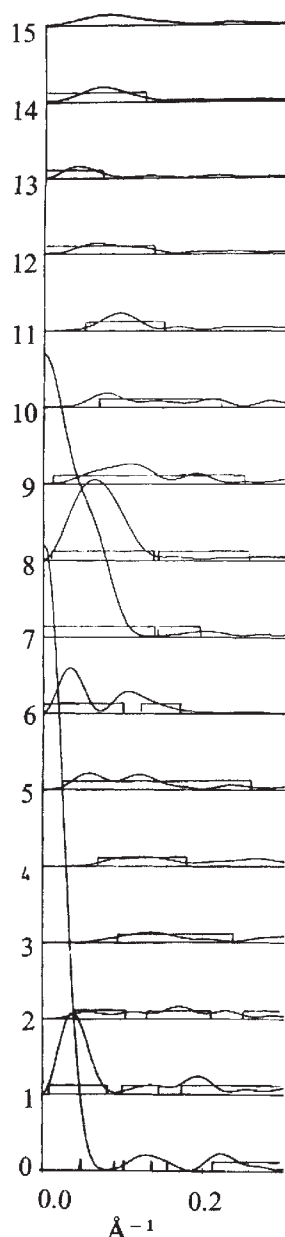


Fig. 2 The cylindrically averaged molecular Fourier transform of the sevenfold TpdA model. The smooth curves indicate the calculated transform. Bragg reflection positions are indicated by vertical bars and the continuous intensity is represented by blocks. Modified atomic scattering factors⁸, to allow for water in the structure, were used in the Fourier transform calculations.

linked to each other by Hoogsteen³ hydrogen bonds, and both adenine and thymine bases are in the anti conformation about the glycosyl bonds. The interaction between bases is thus similar to that between chains 2 and 3 in poly(dT)–poly(dA)–poly(dT) (ref. 4). The adenine–thymine bases are tilted about 9° from perpendicular to the helix axis so that the distance between adjacent adenine–thymine bases is less than the rise per residue. The glycosyl torsion angle O(1')–C(1')–N(9)–C(4) is 176° for the adenine base and the angle O(1')–C(1')–N(1)–C(2) is 220° for the thymine.

Both furanose rings have C3' endo pucker, so the conformation is similar to that in A-DNA and double-helical RNA. Sugar rings with other kinds of pucker did not give such satisfactory models. The conformation of the C5'–O5' bond about the C4'–C5' bond in the link between the nucleosides is in the *gauche-trans* region. Although this is not the preferred conformation for this bond, similar conformations have been

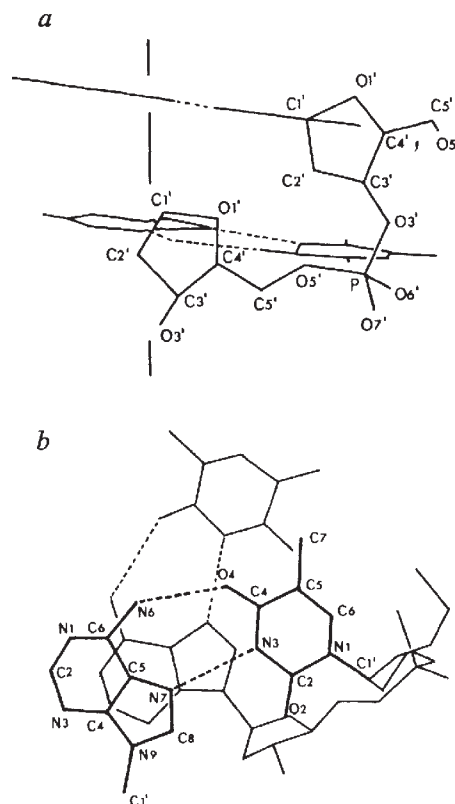
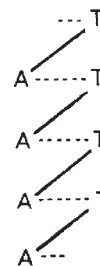


Fig. 3 Projections of one TpdA molecule together with the adenine base of the next molecule above and the thymine base of the next molecule below. *a*, View perpendicular to the helix axis; *b*, view along the helix axis.

observed in some nucleotides⁵⁻⁷. A *gauche-gauche* conformation about the C(4')–C(5') bond consistently gave rise to an unacceptably short contact. Two views of the model are shown in Fig. 3, and a sketch of the model is shown in Fig. 4. There is a large degree of overlap between adenine bases but very little between

Fig. 4 Schematic representation of the model.



the thymine bases. The structure is polar and the fact that the diffraction patterns have discrete reflections only in the inner region of the equator indicates that the 'up' and 'down' arrangement of helices in the specimens is random.

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matters arising

Energy expenditure in children

It was with interest that we read the paper by Griffiths and Payne¹, in which they presented interesting data and then discussed the possible relevance of environmental and genetic factors on the level and nature of the energy balance obtained with the two groups of children.

It is interesting to note that on expressing the mean expenditure and intake data for both groups as a ratio $\times 100$, precisely the same figure ensues

$$\frac{\text{Energy expenditure}}{\text{Energy intake}} \times 100$$

Non-obese	Obese
$\frac{1,508}{1,433} \times 100 =$	$\frac{1,174}{1,115} \times 100 =$
105.23	105.29

Is it not possible therefore, that few overall metabolic differences exist between the 5-yr-old children of these two groups of parents, which by implication, suggests the predominance of environmental factors on these results?

It is surprising that the O group children were found to have lower daily energy intakes than their controls (N group). Therefore it would seem likely that parental pride during the nutritional survey may have had a marked influence.

We are also a little concerned that no comment was made about the data which showed that both groups of children were in negative energy balance, which for 5 yr olds would seem to be unlikely. From our experience SAMI measurements can be misleadingly high unless very precise and immobile electrical contacts are made, and thus background noise is eliminated.

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GRIFFITHS AND PAYNE REPLY—In our view the most important findings in our

study were the differences in total daily energy metabolism, and of resting metabolic rate between the two groups of children.

We agree that the measurements of daily expenditure by the SAMI technique are inherently less reliable than are those of food energy intake, and have offered them as supporting evidence. That is to say, unlike Miller and Otto¹, we regard the equal ratios of expenditure to intake as contributing positively to the evidence for different amounts of energy metabolised daily by the two groups.

Because the differences between the estimations of expenditure and intake (energy balance) are small in relation to the errors inherent in the two measurements, the energy balances do not statistically differ significantly from zero. We are not therefore inclined to attach any biological significance to the fact that they happen, on average, to be negative in both groups.

It is of course possible that one aspect of the behavioural differences between obese and normal people is that the former tend to 'cheat' when attempts are made to measure habitual intake, either their own or their children's. We can only repeat that: the O group children were not themselves measurably overweight so there was no obvious reason for the parents to take pride in suggesting that they eat sparingly, the degree of cooperation and the level of communication with the subjects about the objectives of the study were of necessity, very high, the admittedly less reliable estimates of expenditure closely parallel those of intakes, and show the same magnitude of difference between the groups.

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Benzodiazepines and GABA

BASED on a few experiments on Deiters' neurones and cerebellar Purkinje cells, Steiner and Felix¹ concluded that benzodiazepines antagonise the inhibitory effect of GABA. To understand the mode of action of centrally acting agents, the evidence available from various pharmacological experiments

must be considered and the manifold pitfalls inherent in the single-cell recording and microelectrophoretic techniques taken into account. The conclusion of Steiner and Felix¹ is in contrast to the well documented facts that benzodiazepines: (1) are the most potent antagonists of convulsions induced by picrotoxin and bicuculline^{2–4} (generally accepted to block GABA receptors); (2) enhance presynaptic inhibition in the spinal cord and in the cuneate nucleus^{5,6}; (3) enhance postsynaptic inhibition in the cuneate nucleus⁶ and in the substantia nigra^{2,7} and (4) are mutual antagonists of GABA receptor blockers at inhibitory synaptic processes believed to be mediated by GABA^{5–7}. We think that the conclusion of Steiner and Felix¹ is based on the erroneous interpretation of experimental conditions that were inadequate for the specific problem.

In their experiments on vestibular neurones, they made the implicit assumption that the pulses applied to the cerebellar white substance (in their Fig. 1 either the time calibration is wrong or the rate of stimulation is not that indicated in the legend) released the same amount of GABA within the Deiters' nucleus before and after diazepam. This crucial premise is probably not correct for the following reasons. The stimulating electrode excites Purkinje cell axons directly and, in addition, evokes an unknown number of synaptically evoked discharges of Purkinje cells through the inevitable stimulation of cerebellar afferents. The number of the latter discharges depends on the balance between mono- and disynaptic excitation and the inhibitory influence on Purkinje cells. If diazepam, as we assume, enhances GABA-mediated synaptic inhibition also in the cerebellar cortex, it should reduce the number of Purkinje cell discharges evoked synaptically by the stimulus train, and thus, produce an apparent reduction of the inhibitory effect on Deiters' neurones, which was interpreted by Steiner and Felix¹ as an antagonism of the effect of GABA released within the vestibular nucleus. The apparent reduction by diazepam of the inhibitory effect of iontophoretically applied GABA may be explained in the same way. In the pre-drug period, the depression of Deiters' neurones is achieved by the fixed amount of

exogenous GABA which adds to the endogenous GABA that is released by the continuous activity of Purkinje cells. After diazepam, the spontaneous firing rate of Purkinje cells is markedly reduced^{2,8}. This must result in disinhibition of Deiters' neurones and in the reduction of the total amount of GABA (exogenous plus endogenous) acting on them. The validity of our reasoning could easily be checked experimentally.

Any interpretation of the results of Steiner and Felix¹ on cerebellar Purkinje cells must remain highly speculative when taking into consideration that four of the five types of cerebellar cortical neurones are believed to be GABA-ergic and that the activity of the Purkinje cell is itself inhibited by GABA-ergic interneurons. In the experiments of Steiner and Felix¹ the benzodiazepines did not seem to depress the firing rate of Purkinje cell, whereas in our own experiments^{2,8} several representatives of this class of compounds regularly and markedly reduced the spontaneous discharges and strongly antagonised the opposite effect of bicuculline. We should also like to emphasise that the experiments of Steiner and Felix¹ were performed on anaesthetised animals and a multitude of interactions between anaesthetics and benzodiazepines have been described.

In conclusion, it seems that the findings of Steiner and Felix¹ do not fail to support a facilitating effect of benzodiazepines on GABA-mediated transmission² but rather that they are easily explained by such a mode of action.

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⁷ Keller, H. H., Schaffner, R., and Haefely, W., *Archs Pharmacol.* (in the press).

⁸ Pieri, L., *Experientia* (in the press).

STEINER AND FELIX REPLY—The hypothesis advanced by Haefely *et al.*¹ that benzodiazepines facilitate GABA-responsive neurotransmission is based on the premise that GABA is the proven mediator of inhibition at presynaptic sites (this is still controversial, see refs 2 and 3) as well as on the demonstration

Matters arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

that the action of the presumptive GABA antagonists bicuculline and picrotoxin is reduced by this class of psychotropic drugs. Haefely *et al.*¹ are justified in pointing out that the mode of action of centrally acting drugs is bound to be complex and that more than one level of interaction ought to be considered. They fail to mention, however, that so far, there is no direct experimental support for their contention at any level of organisation tested: Neither their own work, nor that of others cited in support of their view, has directly tested GABA-mediated transmission, and thus all their experimental evidence is circumstantial. It was precisely for this reason that we chose to test their hypothesis at the level of identified Purkinje cells and Deiters' neurones⁴. At both these sites, there is incontrovertible evidence for GABA-mediated inhibition, the extent of which can readily be followed by standard electrophysiological techniques. In this anatomically and functionally well defined system, our results indicated that benzodiazepines inhibit GABA-responsive transmission⁴. We remain convinced that our technique is adequate to study the problem, and welcome this opportunity to answer questions raised by Haefely *et al.*¹ about our approach.

For the orthodromic activation of Deiters' neurones by stimulation of Purkinje cell axons, we deliberately chose supramaximal parameters (trains of three pulses of 250 μ s duration at 500- μ s intervals and an intensity of 0.8 mA). Although we do not refute the view expressed¹ that this type of stimulations is likely to have repercussions on the cerebellar cortex as well as on nerve endings at the level of the Deiters' nucleus, we believe that, as in other biological systems, orthodromically stimulated transmitter release is a direct function of the stimulus applied (and therefore, in our case, a constant); we cannot accept their view that the amount of GABA

released in our experimental conditions is modified, in any important respect, by way of the soma of the Purkinje cells itself. With respect to the methodology used, we concede that in any study using microelectrophoresis, the quantity of exogenous neurotransmitter administered, albeit small in absolute terms, is likely to be in the pharmacological range. Changes in endogenous GABA release would therefore seem negligible compared with the amount of exogenous GABA administered. Haefely *et al.*¹ argue that a drop in endogenous GABA release by Purkinje cells (which, in keeping with their hypothesis, they ascribe to a facilitation of GABA-mediated inhibition by benzodiazepines at this site) could "easily" be distinguished from the fixed amount of exogenous GABA applied; in our view, this would require the direct monitoring of GABA release at Purkinje cell terminals on Deiters' neurones and could not reliably be deduced from the rate of cell firing alone (to our knowledge, such microtechniques are presently beyond our reach).

If Haefely *et al.* were correct in their assumption that benzodiazepines facilitate GABA-mediated inhibition at the Purkinje cell (and so reduce their GABA release), they would also have to concede a similar synergism at the GABA-responsive synapses impinging on the Deiters' neurones themselves; if their argument is taken to its logical conclusion, one would have to postulate that reduced GABA release at this site is counteracted, and possibly cancelled out, by an increase in its biological effect. This assumption, however is not borne out by our findings. Likewise, we consider the absence of benzodiazepine effects on the spontaneous rate of discharge of Purkinje cells as further evidence that our findings are not secondary to changes in the extent of excitation of the cerebellar cortex or the use of anaesthetised animals but are a reflection of drug interaction occurring at the site of study itself.

Our results, as well as those of Gähwiler⁵, obtained in simple well defined systems, indicate a functional antagonism between GABA and benzodiazepines. They do not confirm the notion of a synergism inferred from indirect observations on more complex structures.

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¹ Haefely, W., et al., *Nature*, **263**, 173-174 (1976).

² Ryall, R. W., *Amino Acid Neurotransmitters*, 4 (edit. by Iversen, L. L., Iversen, S. D., and Snyder, S. H.), 101-109 (Plenum, New York, 1975).

³ Schmidt, R. F., *Ergebn. Physiol.*, **63**, 20-101 (1971).

⁴ Steiner, F. A., and Felix, D., *Nature*, **260**, 346-347 (1976).

⁵ Gähwiler, B. H., *Brain Res.*, **107**, 176-179 (1976).

reviews

Marking time

Colin Renfrew

Radiocarbon: Calibration and Prehistory. Edited by Trevor Watkins. Pp. vi+147. (Edinburgh University: Edinburgh, January 1976.) £3.50.

THE tree-ring calibration of radiocarbon dating has had a number of striking archaeological consequences, bringing the historical and radiocarbon calendars for the ancient civilisations of the Old World into closer agreement and pushing the date of some cultures and monuments back much earlier than had been realised.

The intention of the symposium from which this volume springs was to "allow discussion of the subject of correction before a lay audience in terms which they could understand". Most of the contributions succeed well in this modest task. Richard Burleigh, for instance, advocates preliminary calibration for many purposes, while pointing out that for other purposes uncalibrated dates can often legitimately be compared. This view, however, is somewhat over-simplified by the editor: "it is too early to use the curves at present being banded around as calibration curves for radiocarbon dates". Unfortunately the volume went to press before the publication by R. M. Clark of the most statistically sound and reliable of these (*Antiquity*, 49, 252-272, 1975).

The volume as a whole betrays a similar lack of balance. Alongside useful reviews of European dendrochronology by Fletcher and of the problem of irregularities ('kinks') by Ottaway and Ottaway, is a substantial disquisition by McKerrell (53 pages long, more than a third of the total) arguing the very special case that the existing calibration curves are reasonably sound except for the period between 2,500 BC and 1,500 BC (in calendar years).

This view is based on the assessment of radiocarbon and historical dates (the later derived from the Egyptian calendar) for Egypt, the Near East and the Aegean. Unfortunately the archaeology for the Aegean is distinctly shaky (p61) and the treatment of the central European material (Veterov, Madarovce, and so on; p68) might have been more convincing at the hand of a professional archaeologist.

It is surprising that a scientific worker should propose, in the proceedings of a conference designed for laymen, a theory with major geophysical implications which might better first be offered to the critical scrutiny of his physicist colleagues. Moreover, when the theory that calibrated dates in general are sound (with the specific exception of a single millennium), has global implications, would it not be wise to consider the broader consequences outside of Europe?

What begins as a useful introductory handbook rapidly becomes a specialised

and highly controversial critique of calibration, an argument *à thèse* rather than a clarification, on which the comments of competent radiocarbon specialists might have been invited. The editor could usefully have asked himself whether he wanted his book to be a searching professional critique of calibration, or a lucid, non-technical exposition. There is still a need for both. □

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Textbook of ichnology

The Study of Trace Fossils: A Synthesis of Principles, Problems and Procedures in Ichnology. Edited by Robert W. Frey. Pp. xiv+562. (Springer: Berlin and New York, 1975.) DM 141.70; \$58.10.

MANY sedimentary rocks contain fossilised branching structures that a hundred years ago were mistakenly identified as seaweeds and called 'fucoids'. In the 1880's these were shown to be the tracks of animals, and in most parts of the world their study lapsed. But in Germany this was not at all the case. In 1925 Rudolf Richter founded a new school of *Akutuopaläontologie* in which the traces of animals played a vital part in the determination of environment. These early studies led to the establishment of a new and exciting discipline in which palaeontologists were able to postulate behavioural parameters. In this way 'palaeoethology' came to join 'palaeoecology' as a branch of 'geobiology'. In these studies the Germans remained far in advance of the rest of the world for over thirty years; and, indeed, it can still be said that they lead the field, with Professor Adolf Seilacher of Tübingen (who contributes a foreword to this book) as the acknowledged dean.

But at least nowadays the supremacy of our German colleagues in this field has been challenged. The discipline of palaeontology has much changed from the time when it was dominated by morphology and taxonomy. Its bound-

daries have extended, and in 1970 Crimes and Harper edited a remarkably successful symposium on trace fossils which reported the proceedings of an international symposium held in Liverpool. This was followed in 1971 by a field-guide to trace fossils produced by Louisiana State University. It has now been realised all over the world that ichnology plays a quite indispensable role in field studies aimed at interpreting past environments.

The present volume is the only textbook on the subject available in English. It captures well the excitement of a subject which to most of the world is still seen as new. The editor has kept his contributors well disciplined, so that in spite of the fact that it combines the work of no fewer than 28 authors, it retains an admirable cohesiveness as well as being comprehensive. For example, it includes chapters on both plant traces and vertebrate trails (both by William Sarjeant), and concludes with a fascinating section describing two new developments in ichnology—one on experimental methods (By Christopher Elders) and the other describing George Farrow's brilliant techniques for the study of the traces of living animals in present day sediments.

This book will certainly be regarded as an indispensable teaching aid in all future courses dealing with ichnology or palaeoethology, and the editor, Robert Frey, is to be congratulated on the clarity of exposition he has been able to command as well as the logic he

has imparted to the work as a whole. It is all the more to be regretted that the publishers, in spite of their reputation and their experience, have allowed the most appalling errors to appear in the printing of the illustrations. Five of the tables in the book have had all data in them omitted in printing. In a tardy attempt at correcting this error, the publishers have issued with the later copies offered for sale an eight-page 'errata sheet', but with incredible incompetence these have been printed in a way that makes it impossible to substitute them for the faulty pages, as each has been backed on to another, with p472 backing, for example, p516. It can only be hoped that a new printing can put such a stupid blunder right.

P. C. Sylvester-Bradley

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Reaction kinetics

The Organic Chemistry of Electrolyte Solutions. (Interscience Monographs on Organic Chemistry.) By John E. Gordon. Pp. xxi+554. (Wiley-Interscience: New York and London, December 1975.) £15.35.

The Electrolyte Chemistry of Organic Solutions? No, that will not do either; the title is strange and ill-chosen.

The book's aim, according to the author, is to provide the organic chemist with everything he needs to know about electrolyte solutions, to treat all important solution chemistry measurements in one of its three chapters, while avoiding treating in detail aspects accessible through books such as Robinson and Stokes, and Harned and Owen; and therefore to graft on to the traditional physicochemical framework more 'chemical' information lacking in the great books from non-traditional sources such as vibrational and nuclear magnetic resonance spectroscopy. In less pretentious phraseology, to up-date the standard texts by including the important advances over the past 20 years made through the application of spectroscopic techniques. This is not the first attempt to do so in spite of the fly-leaf's claim, nor does the book seem to have been produced any quicker than a multi-authored volume, as the Series Editor claims, for the literature coverage is complete only to mid-1973 and cuts off in August 1974; and the book was not published until the end of 1975.

The three chapters referred to above are called Salt Effects, Ion Solvation and Ion Association, which are not mutually exclusive categories, as

the author realises. Each chapter concludes with a lengthy section discussing applications to kinetic studies, thus revealing the true interest of the author and his intentions. Has he succeeded? To a very large measure, he has. The book is no paste and scissors concoction, but scholarly and nearly always sound in approach and explanation if not in balance and selection of material. For, in discussion of theoretical conductance equations, only the earlier work of Fuoss is discussed, neglecting his later contributions and those of Prue, Pitts and Justice amongst others. Similarly the discussion of pH , under the heading of 'Single ion activity coefficients', returns unnecessarily to the 1920s treatment; and the widely adopted important NBS method is described as "based on yet another convention". Order of introduction of new concepts could have been better conceived. Terms which will surely puzzle the uninitiated when they first appear often receive more detailed explanation many pages later. The book is well illustrated with figures and diagrams drawn from the literature but not always too well associated with textual explanation. Nevertheless, physical organic chemists and others should find much of value in this book as a source book to the extensive literature on electrolyte solutions. It should help to continue to advance the interpretation of reaction kinetics in organic solvents, a subject which has progressed a long way since ion-pairs and solvated ions were first invoked to explain kinetic data when mechanistic explanation was otherwise at a low ebb.

A. K. Covington

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Cell split

Cell Division in Higher Plants. (Experimental Botany: An International Series of Monographs, Vol. 7.) Edited by M. M. Yeoman. Pp. x+542. (Academic: London and New York, April 1976.) £16.50; \$41.

KNOWLEDGE of the cell division process and its control in plants has developed rapidly in recent years from observations made in a diversity of research fields. Dr Yeoman's concept behind this volume was clearly that this scattered material had to be brought together and presented in a developing sequence so that this recent progress could be fully appreciated and the as yet outstanding problems critically identified. The Botany Department at the University of Edinburgh has been one of the main centres of current research on cell division in plants, and

members of this department have obviously played a critical role, under Dr Yeoman's astute editorship, in the realisation of this volume. Professor R. Brown, in an introductory chapter, identifies the problems posed by the organised cell divisions in plant meristems and develops, in a thought-provoking way, models which will clearly guide future research. A group of chapters then deal with the process of cell division both in somatic and reproductive cells. A. F. Dyer contributes two chapters on mitosis which deal in a most lucid way with mitotic cycles and the origin and consequences of mitotic modifications and errors. A parallel chapter by M. D. Bennett (Plant Breeding Institute, Cambridge) deals with meiosis. M. M. Yeoman and P. A. Aitchison summarise our present very limited knowledge of the molecular events of the cell cycle, and Rachel M. Leech (York) our similarly very imperfect understanding of plastid replication.

The second major section of the book considers cell division in relation to the generation of form. The very intensive studies on the root apex are surveyed by F. A. L. Clowes (Oxford). This is followed by chapters on the shoot apex (R. F. Lyndon), leaves (J. E. Dale) and cambium (I. D. J. Phillips, Exeter). W. A. Jensen (Berkeley, California) contributes a disappointing short chapter on cell division in embryo development. This section of the book is completed by an interesting survey of unorganised cell division and morphogenesis in tissue and cell cultures by A. W. Davidson, P. A. Aitchison and M. M. Yeoman.

A final section headed Summary and Perspectives and written by Dr Yeoman suffers from its extreme brevity (4 pages) so that it is little more than a list of central themes and a signposting to the various fields of research (covered in the preceding chapters) which are contributing to these themes. Some critical discussion of perspectives would have been of real value here.

Setting aside these minor blemishes the volume is timely, well conceived, and scholarly written. It fills a real gap in botanical literature. In general the chapters are uniform and appropriate in standard so that they will be of value to established scientists looking for a well documented survey but equally appropriate for advanced undergraduate reading. The authors and press are to be congratulated on the excellent plates and informative line diagrams many of which have been specially prepared for the volume.

H. E. Street

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Energy past and energy present

Man, Energy, Society By Earl Cook. Pp. xi+478. (Freeman: San Francisco and Reading, May 1976.) Cloth £10.40; paper £4.50.

THE availability of energy is clearly a key element in the history of civilisations, the size, form and behaviour of societies and the choices open to them. Following in the steps of Fred Cottrell's remarkable *Energy and Society* (1955), the geologist and geographer Earl Cook here re-explores this fascinating set of connections. The result is a valuable, broad introduction to 'energy' in a historical, social, geographical and economic setting. The sweep is immense, ranging from the emergence of agriculture to future energy alternatives of varying socio-political stability. The approach is often refreshingly catholic: thus, a major section on the global distribution of energy resources starts with arable and pasture land; food (and its getting) commands as much attention as the major fossil fuels; the ethics of resource depletion and the sociology of control of energy use are discussed as thoroughly as the usual technical and economic topics. One consequence is to make the book a valuable source of inaccessible statistics and data, such as the efficiency of early machines; the total system energetics of transport, food production, mining and other industrial activities; and the rising energy and other costs of winning lower grade mineral and fuel resources.

Although useful up to a point, any uni-dimensional view of the world can all too easily be taken too far into empty truisms and gross overstatement. Cook all too often falls into these traps, walking dangerously close to a pure 'energy theory of value'. Statements such as "a doubling of living level requires approximately a doubling of available energy" (p190); that if energy use per capita is not increasing then economic development is reversing (p218); and that the gap between developed and underdeveloped regions in per capita energy use is widening (p18), underpin many of the book's theses, yet are demonstrably false. Nor is it exactly helpful, in the absence of any sustained discussion of overt political factors in the control of energy or of society, to come across remarks such as "high energy man seeks diversion in sports, business, drugs, sex, rioting, and crime" (p221), or that the Crusades could not have happened without the mobilisation of energy surpluses in Northern Europe "reflected in horses, armour and men"

(p195). Yet read with a wary eye and a few pinches of salt, this is nevertheless a valuable book as both a thought-provoker and source of information.

Energy Resources and Supply. By J. T. McMullan, R. Morgan and R. B. Murray. Pp. xii+508. (Wiley-Interscience: London and New York, March 1976.) £12.50; \$27.50.

FEW of the 'broad survey' books on energy that are now flooding the market focus with such admirable clarity, thoroughness and wide scope as does this on the hard-nosed physics and engineering of the subject. Aimed at university courses and the more numerate specialists, the emphasis is predominantly on techniques and their physicochemical basis—in many ways a refreshing change that helps fill an important gap. There is also a strong bias towards the 'mainstream' supply and conversion technologies, with about half the book devoted to resources, extraction, processing and characteristics of the fossil fuels and the nuclear fuel cycle, both fission and fusion. Opening chapters survey the energetics of the biosphere-atmosphere system and photosynthesis, whereas there are relatively sketchy chapters on solar energy, other renewable sources, storage, energy-in-use and (with proper enthusiasm) the heat pump.

Plant ecophysiology

Physiological Plant Ecology. By W. Larcher. Translated by M. A. Biederman-Thorson. Pp. xiv+252. (Springer: Berlin and New York, 1975.) DM 46; \$18.90.

IN revising and translating into English the original German text *Ökologie der Pflanzen*, the opportunity has been taken to adopt a more accurate title, which is supported in the Preface by an adequate description of the subject matter and aims of the book. Ecologists urgently seeking physiological information relevant to field situations for teaching and research purposes, invariably have to search a voluminous literature. Professor Larcher is to be congratulated on making this task so much lighter, by producing a concise account of many important aspects of plant ecophysiology.

Inevitably, the topics covered reflect the authors' own research interests and the limitations of space and cost. The approach adopted of portraying the development of the subject in the choice of illustrations and tables is ex-

The coverage and density of information is impressive. Yet there are curious gaps. For example, there is much about peat as a large 'fossil' resource but nothing about the vast annually renewed fuel sources in (tropical) estuarine biomass; in a long section on nuclear reactors there is the scantiest reference to comparative efficiencies of fuel use; the account of solar collectors bristles with algebra but there is hardly a word about overall system design or integration of solar with other ideas (including storage and heat pumps), the keys to success in this area. More seriously, there is such a determined avoidance of almost everything but physics and engineering that the real world sometimes seems light years away. There is almost no mention even of costs, and the broader economic and social aspects of energy are confined to a single chapter of six pages. The total misunderstanding of the energy situation, needs and constraints of the undeveloped world, revealed in the final pages, is no less than appalling in a book which in many ways deserves to become a basic text for undergraduate and graduate students in the physical, engineering and environmental sciences.

Gerald Leach

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tremely interesting. Much of the material is new and valuable syntheses have been made in a number of tables, yet in certain sections, especially those on nitrogen and mineral elements, the treatment is somewhat out-of-date. Under the heading 'Dry-matter Production' (p63) the section on growth analysis would greatly benefit by additional references to recent alternative articles and texts. There are very few obvious errors in the text: (cf p000) should be (cf p134) on p54 and (cf p000) following Id=direct solar radiation (p191) should be omitted from this revised translation. I would prefer to see Leguminosae used on pp94, 101, 115 and 119 instead of Fabaceae.

The book is a valuable source of reference to articles normally hidden away in the German literature and it is to be regretted that the current rates of exchange will put it beyond the pockets of most undergraduates.

K. Taylor

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obituary

Jerome Vinograd was born February 9, 1913 in Milwaukee, Wisconsin, and died July 3, 1976. After undergraduate work at the University of Minnesota, he studied colloid chemistry with H. Freundlich from 1931 to 1935, first in Berlin then in London. He completed his doctoral work with J. W. McBain at Stanford from 1937 to 1939 and was associated with the Shell Development Co. from 1941 to 1951 where he worked mainly on problems of surface films and colloids. At that time, he gave up a secure industrial career and came to the California Institute of Technology because of his intense desire to work in the exciting new area of molecular biology. His early work at Caltech dealt mainly with the physical chemistry of proteins.

His first outstanding contribution to molecular biology was the invention, in collaboration with Meselson and Stahl, of the method of equilibrium density gradient centrifugation of nucleic acids in caesium chloride solutions. This method has been elaborated in Vinograd's and other laboratories to take advantage of the effects on buoyant density of base composition, denaturation, alkaline titration, binding of metal ions and of dyes, and hydration. All told, equilibrium buoyant banding has been one of the key techniques in the explosive development of the nucleic acid side of molecular biology since the mid 1950s.

Vinograd's outstanding work in the past 12 years has been in the study of closed circular DNA. The distinction between supercoiled closed circles and open circles was recognised in 1965. The ethidium bromide method of buoyant banding for the isolation of closed circular DNA was invented in 1967. This method, which makes it possible to analyse for and to isolate closed circular DNA in the presence of massive quantities of linear and open circular DNA, is widely used, and is one of the main reasons why there has been such exciting progress in the study of

closed circular DNAs in different kinds of cells.

Later work dealt with the properties of mitochondrial DNA from malignant cells and with the study of the replication of mitochondrial DNA, initiated by the discovery of D-loops in Vinograd's laboratory. More recently his students have made several important contributions to the studies of the properties of the nicking and closing enzyme which relaxes supercoiled DNA while leaving it covalently closed. The Vinograd group as well as the groups of Keller at Cold Spring Harbor and Wang at Berkeley observed that one can use gel electrophoresis and relaxation by an enzyme to count the number of turns in a closed circular DNA, and that there is a Boltzmann distribution in the number of superturns in molecules which are closed, under equilibrium conditions.

Vinograd was, by early training and in his approach to molecular biology, a physical chemist. He had a flair for recognising when an anomalous and unexplained observation, if subjected to fundamental physical chemical analysis, would lead to an unexpected and important result in molecular biology.

I have seen the special Vinograd touch many times. A simple example is the development of the velocity band centrifugation method by Vinograd, Bruner, Kent and Weigle in 1963. Shortly before, Jean Weigle had discovered that he could layer a dilute solution of viruses in aqueous buffer on to a sucrose solution (not a gradient) in a centrifuge tube, centrifuge and get a good sharp virus band. Vinograd was puzzled as to why the band was sharp, and reasoned that there must be some effect providing convective stability. He recognised that there had to be a self-generating (by diffusion) density gradient to provide convective stability; a careful study then led to the development of this general and widely used method.

I remember vividly a seminar at Caltech in late 1966 or early 1967 when J. B. LePecq described his studies on the binding of ethidium bromide (Etd Br) to DNA. An important point was that, unlike the common acridine dyes, there was a reasonable amount of binding at high salt concentration. Vinograd pointed out at the discussion which immediately followed that if the dye ion bound in 6 M CsCl it would cause a large shift in the buoyant density of the DNA because it would displace a Cs⁺ ion. Experiments by W. R. Bauer (then a graduate student) showed that Etd Br would indeed decrease the buoyant density of DNA, but that the shift was much larger for a relaxed than for a closed circular DNA. On learning of this result, Vinograd immediately perceived the correct explanation in terms of the topological constraint on the unwinding of closed circular DNA, thus leading to the development of an extremely useful method for isolating closed circular DNA, as well as for studying the free energy supercoiling.

He suffered a major heart attack in 1954 and a second one in 1969. He accepted the resulting restrictions on his overall activities philosophically. But his condition did not affect the intensity with which he devoted himself to his scientific work. I remember him through all these years being in the laboratory regularly on evenings and weekends discussing and analysing experimental data in painstaking detail with his students, seeking a fundamental explanation for unexpected and unexplained results. His students and colleagues will miss his penetrating insight into technical problems and his wise counsel on general policy issues.

Vinograd was made a member of the National Academy of Sciences in 1968, and received numerous awards. He is survived by his wife Dorothy, and his two daughters Julie and Deborah by a previous marriage.

Norman Davidson

Person to Person

The Beilby Medal and Prize will be made to a British scientist for work in any field related to the special interests of Sir George Beilby (chemical engineering, fuel technology or metallurgy). Apply to The Convener of the Administrators, Sir George Beilby Memorial Fund, The Royal Institute of Chemistry, 30 Russell Square, London, WC1B 5DT.

The Commission of the European Communities is sponsoring travelling Fellowships for obstetricians, neonatologists and research workers who are actively engaged in perinatal monitoring. The successful applicants will spend three weeks in a centre for perinatal monitoring within the EEC, outside their own country. Applications to Commission of the European Communities, Directorate-General Research, Science and

Education, X11/C-1, 200, rue de la Loi, 1049 Brussels.

Address and details of the work of Rose Selavy on stellar lightning (ref. Brecher, K., *Nature*, **261**, 542; 1976) would be appreciated by Mr E. W. Crew, 26 St David's Drive, Broxbourne, Herts, UK.

There will be no charge for this service. Send items (not more than 60 words) to Martin Goldman at the London office. The section will include exchanges of accommodation, personal announcements and scientific queries. We reserve the right to decline material submitted. No commercial transactions.